(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
19 April 2001 (19.04.2001)

PCT

(10) International Publication Number
WO 01/27258 A2

(51) International Patent Classification[7]: C12N 15/00

(21) International Application Number: PCT/US00/28578

(22) International Filing Date: 13 October 2000 (13.10.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/159,467 13 October 1999 (13.10.1999) US

(71) Applicant: THE UNIVERSITY OF GEORGIA RESEARCH FOUNDATION, INC. [US/US]; 632 Boyd Graduate Studies Research Center, Athens, GA 30602-7411 (US).

(71) Applicants and
(72) Inventors: EITEMAN, Mark, A. [US/US]; 111 Riverbottom Way, Athens, GA 30606 (US). ALTMAN, Elliot [US/US]; 210 Great Oak Drive, Athens, GA 30605 (US). GOKARN, Ravi, R. [IN/US]; 14300 34th Avenue North, #209, Plymouth, MN 55447 (US).

(74) Agent: SANDBERG, Victoria, A.; Mueting, Raasch & Gebhardt, P.A., P.O. Box 581415, Minneapolis, MN 55458-1415 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/27258 A2

(54) Title: HIGH YIELD PROTEIN EXPRESSION SYSTEM AND METHODS

(57) Abstract: Increasing pyruvate carboxylase activity in various protein expression systems results in enhanced protein yields.

HIGH YIELD PROTEIN EXPRESSION SYSTEM AND METHODS

Commercial production of recombinant proteins for industrial and medical use has increased significantly in recent years. For example, conventional chemical-based synthetic processes are increasingly being replaced by large-scale processes using enzyme catalysis (biocatalysis). The food and detergent industries are two major industries that make use of large-scale biological processes. Enzymes important in the dairy industry include rennet, lactase, papain and pectinesterases; in starch processing, α-amylase, glucoamylase and glucose isomerase; in the detergent industry, protease, lipase and amylase; in textiles, amylase; and in pulp and paper, cellulases. The market for diagnostic and therapeutic proteins and peptides is also expanding. Major peptide drugs include erythropoietin, insulin, granulocyte colony stimulating factor, human growth hormone and interferon.

Although costs for recombinant production of industrial and therapeutic proteins and peptides include those associated with both fermentation and purification processes, the limiting factor is the concentration of active protein or peptide that can be achieved from the fermentation process. Thus, a method for increasing the yield of recombinant proteins would result in significant cost savings to the industry.

Escherichia coli is the most widely used organism for recombinant protein production. It is well-characterized, fast and inexpensive to grow, and relatively easy to alter genetically. Some strains can produce as much as 30 percent of their total protein as the expressed gene product. Proteins produced from recombinant *E. coli* include insulin, interferons, growth hormones, interleukins, hydrolases, reductase, and transferases.

In order to generate cell mass and produce proteins, all cells must catabolize glucose or other carbohydrates to produce energy and key metabolites. These carbohydrates are processed via glycolysis and converted to phosphoenolpyruvate (PEP). PEP is converted into two important metabolic

intermediates, pyruvate and oxaloacetate (see Fig. 1). Pyruvate is then converted to acetyl-CoA. In the presence of oxygen, as is typically the case for recombinant protein production, acetyl-CoA and the 4-carbon metabolite oxaloacetete combine to yield the 6-carbon metabolite citrate, thereby fueling the tricarboxylic acid (TCA) cycle and providing energy and key metabolites for the cell (see Fig. 1). Alternatively, acetyl CoA can undergo a two-step process to form acetate, while oxaloacetate can be used to generate several amino acids.

Prior attempts to increase the efficiency of large-scale recombinant protein synthesis in *E. coli* have focused on reducing the amount of acetate accumulated during fermentations, as acetate accumulation has been suspected of being a limiting factor in achieving high protein yields. However, despite vigorous research efforts in this area, this approach has not let to significant advances in recombinant protein production. Continued advances in recombinant protein production techniques are highly desired and actively sought by the emerging biological processing industry. Clearly, a new approach toward increasing protein yield in large scale fermentations is needed.

Summary of the Invention

Pyruvate carboxylase is a biotin-dependent enzyme which can accomplish anaplerotic reactions by converting pyruvate and carbon dioxide into oxaloacetate. This enzyme has been found in many different cell types ranging from bacteria to human. It has been surprisingly found that increasing pyruvate carboxylase activity within a host cell results in an enhancement of polypeptide production. Thus, the invention involves increasing pyruvate carboxylase activity within a host cell that produces a protein or peptide. The enhancement in polypeptide production by the invention is fully expected to be independent of the type of host cell, the source of the pyruvate carboxylase gene, and the nature of the protein or peptide expressed. The present invention thus represents an unprecedented advance in recombinant protein engineering; virtually any cell that is used to produce proteins or peptides can be genetically engineered in accordance with the invention to improve protein or peptide yield.

The present invention thus includes a method for enhancing protein or peptide production in a host cell used for protein production. The method involves metabolically engineering the host cell by introducing into the cell a native (i.e., endogenous) and/or foreign (i.e., heterologous) nucleic acid fragment that functionally encodes a pyruvate carboxylase so as to overproduce pyruvate carboxylase in the host cell, relative to a cell that has not been so engineered. Surprisingly and advantageously, engineering the host cell to overproduce pyruvate carboxylase enhances production of the protein or peptide of interest. Alternatively, the DNA of a cell that endogenously expresses a pyruvate carboxylase can be mutated to increase expression of the native pyruvate carboxylase gene and hence the pyruvate carboxylase enzyme so as to cause the cell to exhibit enhanced protein or peptide production.

Overexpression of pyruvate carboxylase is preferably effected by transforming a host cell with a DNA fragment encoding a pyruvate carboxylase that is derived from an organism that endogenously expresses pyruvate carboxylase, such as *Rhizobium etli, Corynebacterium glutamicum, Methanobacterium thermoautotrophicum*, or *Pseudomonas fluorescens*. Pyruvate carboxylase can be expressed within the metabolically engineered cell from an expression vector, or alternatively from a DNA fragment that has been chromosomally integrated into the host cell's genome. The host cell is not limited in any way and can be any type of cell that is used for the production of proteins or peptides. In a particularly preferred embodiment of the invention, the metabolically engineered cell is a bacterial cell such as an *E. coli* or *Bacillus subtilis* cell, a yeast cell, a plant cell, an insect cell, or a mammalian cell such as a mouse or human cell.

The present invention further includes a method for making a protein or peptide. The method for making the protein or peptide includes providing a metabolically engineered cell that overexpresses pyruvate carboxylase followed by culturing the metabolically engineered cell to cause increased expression of the protein or peptide. Optionally, the method includes transforming the host cell with a nucleic acid fragment containing a nucleotide sequence encoding a pyruvate carboxylase enzyme to yield the metabolically engineered cell. Also

optionally, the protein or peptide can be isolated from the cell. In an alternative method, the DNA of a host cell that endogenously expresses a pyruvate carboxylase can be mutated to alter transcription of the native pyruvate carboxylase gene so as to cause overproduction of the native enzyme.

Also provided by the invention is a novel protein expression system. The protein expression system includes any cell or cell culture that is useful for the expression of proteins or peptides, wherein the cell or cells have been modified to overexpress pyruvate carboxylase in accordance with the invention, thereby enhancing protein or peptide production. The protein expression system of the invention is capable of producing higher yields of proteins or peptides than the expression system utilizing the analogous cell or cell culture that does not overexpress pyruvate carboxylase.

The invention further provides a metabolically engineered cell for use in the production of a protein or peptide, wherein the cell has been metabolically engineered to overexpress PEP carboxylase so as to cause enhanced production of the protein or peptide compared to a comparable cell that does not overexpress PEP carboxylase. Preferably the cell is one that does not use PEP to effect transport of glucose into the cell. Also provided is a method for making a protein or peptide that includes culturing a metabolically engineered cell that overexpresses PEP carboxylase for a time and under conditions to produce the protein or peptide.

Brief Description of the Figures

Figure 1 shows an aerobic metabolic pathway in *E. coli* depicting glycolysis, the TCA cycle, and biosynthesis of oxaloacetate-derived biochemicals; dashed lines signify that multiple steps are required to biosynthesize the compound while solid lines signify a one-step conversion; the participation of PEP in glucose uptake is shown by a light line; the pathway as shown is not stoichiometric, nor does it include cofactors.

Figure 2 is a graph showing kinetic analysis of pyruvate carboxylase activities for MG1655 pUC18 (○) and MG1655 pUC18-*pyc* (●) with respect to pyruvate.

Figure 3 is a graph showing the effects of increasing aspartate concentrations on the activity of pyruvate carboxylase.

Figure 4 is a graph showing kinetic analysis of pyruvate carboxylase with respect to ATP and ADP; pyruvate carboxylase activity was determined in the absence of ADP (●) and in the presence of 1.5 mM ADP (○).

Figure 5 is a petri plate showing growth of a *ppc* null *E. coli* strain which contains either pUC18 or the pUC18-*pyc* construct on minimal media that utilizes glucose as a sole carbon source.

Figure 6 shows an anaerobic pathway in *E. coli* depicting glycolysis and biosynthesis of selected oxaloacetate-derived biochemicals; the participation of PEP in glucose uptake is shown by the dashed line; the pathway as shown is not stoichiometric, nor does it include all cofactors.

Figure 7 is a graph showing the effect of nicotinamide nucleotides on pyruvate carboxylase activity: NADH (○), NAD^+(□), NADPH (▵) and $NADP^+$ (◇).

Figure 8 is a graph showing the growth pattern and selected fermentation products of wild-type strain (MG1655) under strict anaerobic conditions in a glucose-limited (10 g/L) medium; concentrations of glucose (●), succinate (■), lactate (○), formate (□) and dry cell mass (▵) were measured.

Figure 9 is a graph showing growth pattern and selected fermentation products of wild-type strain with pUC18 cloning/expression vector (MG1655/pUC18) under strict anaerobic conditions in a glucose-limited (10 g/L) medium; concentrations of glucose (●), succinate (■), lactate (○), formate (□) and dry cell mass (▵) were measured.

Figure 10 is a graph showing growth pattern and selected fermentation products of wild-type strain with *pyc* gene (MG1655/pUC18-*pyc*) under strict anaerobic conditions in a glucose-limited (10 g/L) medium; concentrations of

glucose (●), succinate (■), lactate (○), formate (□) and dry cell mass (▵) were measured.

Figure 11 is a graph showing growth pattern and threonine production in the threonine producing strain βIM-4 (ATCC 21277) containing either pTrc99A or pTrc99A-*pyc* under strict aerobic conditions in a glucose-limited (30 g/L) medium; optical density in the pTrc99A containing strain (○), optical density in the pTrc99A-pyc containing strain (□), threonine concentrations in the pTrc99A containing strain (●), and threonine concentrations in the pTrc99A-pyc containing strain (■) were measured.

Figure 12 is a graph depicting production of β-galactosidase from *E. coli* MG1655 pTrc99A-*pyc* pACYC-*lacZ* (●) and *E. coli* MG1655 pTrc99A pACYC-*lacZ* (○) grown in 100 mL shake flasks.

Figure 13 is a graph depicting production of catechol 2,3-dioxygenase from *E. coli* MG1655 pTrc99A-*pyc* pACYC-*xylE* (●) and *E. coli* MG1655 pTrc99A pACYC-*xylE* (○) grown in 100 mL shake flasks.

Figure 14 is a graph depicting cell mass concentration from *E. coli* MG1655 pTrc99A-*pyc* pACYC-*lacZ* (●) and *E. coli* MG1655 pTrc99A pACYC-*lacZ* (○), together with production of β-galactosidase for *E. coli* MG1655 pTrc99A-*pyc* pACYC-*lacZ* (▴) and *E. coli* MG1655 pTrc99A pACYC-*lacZ* (▵), grown in 2 L fermenters using sodium hydroxide for pH control.

Figure 15 is a graph depicting glucose concentration from *E. coli* MG1655 pTrc99A-*pyc* pACYC-*lacZ* (●) and *E. coli* MG1655 pTrc99A pACYC-*lacZ* (○), together with acetate concentration for *E. coli* MG1655 pTrc99A-*pyc* pACYC-*lacZ* (▴) and *E. coli* MG1655 pTrc99A pACYC-*lacZ* (▵), grown in 2 L fermenters using sodium hydroxide for pH control.

Figure 16 is a graph depicting cell mass concentration from *E. coli* MG1655 pTrc99A-*pyc* pACYC-*lacZ* (●) and *E. coli* MG1655 pTrc99A pACYC-*lacZ* (○), together with production of β-galactosidase for *E. coli* MG1655 pTrc99A-*pyc* pACYC-*lacZ* (▴) and *E. coli* MG1655 pTrc99A pACYC-*lacZ* (▵) grown in 2 L fermenters using sodium carbonate for pH control.

Figure 17 is a graph depicting glucose concentration from *E. coli*

MG1655 pTrc99A-*pyc* pACYC-*lacZ* (●) and *E. coli* MG1655 pTrc99A pACYC-*lacZ* (○), together with acetate concentration for *E. coli* MG1655 pTrc99A-*pyc* pACYC-*lacZ* (▲) and *E. coli* MG1655 pTrc99A pACYC-*lacZ* (△), grown in 2 L fermenters using sodium carbonate for pH control.

Figure 18 is a graph depicting fermentation of *E. coli* MG 1655 pTrc99A pACYC-*lacZ* on defined glucose media (Media C2, Example IX). Symbols: (●) glucose, (■) β-galactosidase, (○) optical density, (△) acetate.

Figure 19 is a graph depicting fermentation of *E. coli* MG1655 pTrc99A-*pyc* pACYC-*lacZ* on defined glucose media (Media C2, Example IX). Symbols: (●) glucose, (■) β-galactosidase, (○) optical density, (△) acetate.

Detailed Description

As already noted, previous efforts to increase protein yields in recombinant systems have focused on reducing acetate production. The present invention takes an entirely different approach, focusing instead on metabolically engineering a cell to upregulate an anaplerotic reaction that replenishes TCA cycle intermediates by overproducing pyruvate carboxylase. The success achieved by this approach was highly unexpected. First, metabolic regulation of carbon flow in a cell is strictly regulated. Various metabolic engineering strategies have been pursued, with little success, in an effort to overcome the network rigidity that surrounds carbon metabolism. For example, overexpression of the native enzyme PEP carboxylase in *E. coli* was shown to increase the carbon flux towards oxaloacetate (C. Millard et al., Appl. Environ. Microbiol., 62, 1808-1810 (1996); W. Farmer et al, Appl. Env. Microbiol., 63, 3205-3210 (1997)); however, such genetic manipulations also cause a decrease in glucose uptake (P. Chao et al., Appl. Env. Microbiol., 59, 4261-4265 (1993)), since PEP is a required cosubstrate for glucose transport via the phosphotransferase system. In general, carbon flux towards the important TCA cycle intermediate oxaloacetate remains constant regardless of system perturbations (J. Vallino et al., Biotechnol. Bioeng., 41, 633-646 (1993)).

Second, only ten amino acids (glutamate, arginine, proline, glutamine, aspartate, asparagine, lysine, methionine, threonine and isoleucine) are produced from TCA cycle intermediates, thus production of proteins and peptides (which use all 20 amino acids) would not be expected to increase even if carbon could be successfully diverted into the TCA cycle.

Thus, contrary to what would have been predicted by the established scientific understanding in this field, we have discovered that overproduction of pyruvate carboxylase, which catalyzes the reaction of pyruvate to oxaloacetate, replenishes carbon to the TCA cycle so that the TCA cycle components remain at high levels, thereby ensuring continued cell growth and protein production regardless of whether acetate accumulates in the culture or not.

The terms "pyruvate carboxylase" and "pyruvate carboxylase enzyme" mean an enzyme that has pyruvate carboxylase activity, i.e., that is able to catalyze carboxylation of pyruvate to yield oxaloacetate. The term "pyruvate carboxylase" thus includes naturally occurring pyruvate carboxylase enzymes, along with fragments, derivatives, or other chemical, enzymatic or structural modifications thereof, including enzymes encoded by insertion, deletion or site mutants of naturally occurring pyruvate carboxylase genes, as long as pyruvate carboxylase activity is retained. Pyruvate carboxylase activity is conveniently measured by the coupled method of J. Payne et al. (J. Gen. Microbiol. 59:97-101 (1969)). The term "pyruvate carboxylase gene" means a gene that functionally encodes a pyruvate carboxylase enzyme. A protein or peptide that is "functionally encoded" by a gene or other nucleic acid is one which, when introduced into a host cell, is capable of being expressed by the host cell. For example, the nucleic acid encoding the protein or peptide can include or encode transcriptional and translational regulatory elements such as promoters, operators, enhancers, termination signals, transcription start and stop codons, and the like, that permit constitutive or inducible transcription and translation of the encoded protein. An enzyme is "overexpressed" or "overproduced" in a host cell of the invention when the enzyme is expressed in the host cell at a level higher than the level at which it is expressed in a comparable wild-type cell. In cells that do not endogenously express a particular enzyme, any level of

expression of that enzyme in the cell is deemed an "overexpression" or "overproduction" of that enzyme for purposes of the present invention.

Genetically engineered cells are referred to herein as "metabolically engineered" cells when the genetic engineering is directed to disruption or alteration of a metabolic pathway so as to cause a change in the metabolism of carbon. The pyruvate carboxylase that is overexpressed by a metabolically engineered cell in accordance with the invention can be either endogenous or heterologous. A "heterologous" enzyme is one that is encoded by a nucleotide sequence that is not normally present in the cell. For example, a bacterial cell that has been transformed with and expresses a gene from a different species or genus that encodes a pyruvate carboxylase contains a heterologous pyruvate carboxylase. The heterologous nucleic acid fragment may or may not be integrated into the host genome. Pyruvate carboxylase enzymes and, in some cases, genes that have been characterized include human pyruvate carboxylase (GenBank K02282; S. Freytag et al., J. Biol. Chem., 259, 12831-12837 (1984)); pyruvate carboxylase from *Saccharomyces cerevisiae* (GenBank X59890, J03889, and M16595; R. Stucka et al., Mol. Gen. Genet., 229, 305-315 (1991); F. Lim et al., J. Biol. Chem., 263, 11493-11497 (1988); D. Myers et al., Biochemistry, 22, 5090-5096 (1983)); pyruvate carboxylase from *Schizosaccharomyces pombe* (GenBank D78170); pyruvate carboxylase from *R. etli* (GenBank U51439; M. Dunn et al., J. Bacteriol., 178, 5960-5070 (1996)); pyruvate carboxylase from *Rattus norvegicus* (GenBank U81515; S. Jitrapakdee et al., J. Biol. Chem., 272, 20522-20530 (1997)); pyruvate carboxylase from *Bacillus stearothermophilus* (GenBank D83706; H. Kondo, Gene, 191, 47-50 (1997); S. Libor, Biochemistry, 18, 3647-3653 (1979)); pyruvate carboxylase from *P. fluorescens* (R. Silvia et al., J. Gen. Microbiol., 93, 75-81 (1976); pyruvate carboxylase from *M. thermoautotrophicum* (B. Mukhapodhyay et al., J. Biol. Chem., 273, 5155-5166 (1998)) and pyruvate carboxylase from *C. glutamicum* (GenBank Y09548).

Preferably, the pyruvate carboxylase that is overexpressed by the metabolically engineered cell in accordance with the invention is derived from either *R. etli* or *P. fluorescens*. The pyruvate carboxylase in *R. etli* is encoded by

the *pyc* gene (M. Dunn et al., J. Bacteriol., 178, 5960-5970 (1996)). The *R. etli* enzyme is classified as an α4 pyruvate carboxylase, which is inhibited by aspartate and requires acetyl CoA for activation. Members of this class of pyruvate carboxylases might not seem particularly well-suited for use in the present invention, since redirecting carbon flow from pyruvate to oxaloacetate would be expected to cause reduced production of acetyl CoA, and increased production of aspartate, both of which will decrease pyruvate carboxylase activity. However, expression of *R. etli* pyruvate carboxylase in a bacterial host was found to be effective to increase production of oxaloacetate and its downstream metabolites (Examples II and IV). Moreover, this can be accomplished without adversely affecting glucose uptake by the host (Example III), which has been the stumbling block in previous efforts to divert carbon to oxaloacetate by overexpressing PEP carboxylase (P. Chao et al., Appl. Env. Microbiol., 59, 4261-4265 (1993)).

In a particularly preferred embodiment, the metabolically engineered cell expresses an α4β4 pyruvate carboxylase. Members of this class of pyruvate carboxylases do not require acetyl CoA for activation, nor are they inhibited by aspartate, rendering them particularly well-suited for use in the present invention. *P. fluorescens* is one organism known to expresses an α4β4 pyruvate carboxylase. The metabolically engineered cell of the invention therefore is preferably one that has been transformed with a nucleic acid fragment isolated from *P. fluorescens* which contains a nucleotide sequence encoding a pyruvate carboxylase expressed therein, more preferably the pyruvate carboxylase isolated and described in R. Silvia et al., J. Gen. Microbiol., 93, 75-81 (1976).

Preferably, the metabolically engineered cell of the invention overexpresses pyruvate carboxylase. Stated in another way, the metabolically engineered cell preferably expresses pyruvate carboxylase at a level higher than the level of pyruvate carboxylase expressed in a comparable wild-type cell. This comparison can be made in any number of ways by one of skill in the art and is done under comparable growth conditions. For example, pyruvate carboxylase activity can be quantified and compared using the method of Payne and Morris (J. Gen. Microbiol., 59, 97-101 (1969)). The metabolically engineered cell that

overexpresses pyruvate carboxylase will yield a greater activity than a wild-type cell in this assay. In addition, or alternatively, the amount of pyruvate carboxylase can be quantified and compared by preparing protein extracts from the cells, subjecting them to SDS-PAGE, transferring them to a Western blot, then detecting the biotinylated pyruvate carboxylase protein using detection kits which are commercial available from, for example, Pierce Chemical Company (Rockford, IL), Sigma Chemical Company (St. Louis, MO) or Boehringer Mannheim (Indianapolis, IN) for visualizing biotinylated proteins on Western blots. In some suitable host cells, pyruvate carboxylase expression in the non-engineered, wild-type cell may be below detectable levels.

The metabolically engineered cell utilized in the invention is not limited in any way to any particular type or class of cell. It can be a eukaryotic cell or a prokaryotic cell, without limitation; it can thus include a cell of a bacterium, a plant, a protist (such as a protozoan or an algae), a fungus (such as yeast), or an animal. An animal cell includes, for example, the cell of a vertebrate or an invertebrate, such as an insect cell or a mammalian cell, preferably a mouse or a human cell. A bacterial cell includes, for example, the cell of a bacterium or an archaebacterium. Preferably, the cell is a bacterial cell, a yeast cell, a plant cell, an insect cell, or a mammalian cell. Particularly preferred bacterial cells are *E. coli* cells and *B. subtilis* cells. Preferred mammalian cells are human cells and mouse cells.

Many organisms can synthesize oxaloacetate from either PEP via the enzyme PEP carboxylase, or from pyruvate via the enzyme pyruvate carboxylase. Representatives of this class of organisms include *C. glutamicum*, *R. etli*, *P. fluorescens*, *Pseudomonas citronellolis*, *Azotobacter vinelandii*, *Aspergillus nidulans*, and rat liver cells. Other organisms cannot synthesize oxaloacetate directly from pyruvate because they lack the enzyme pyruvate carboxylase. *E. coli*, *Salmonella typhimurium*, *Fibrobacter succinogenes*, and *Ruminococcus flavefaciens* are representatives of this class of organisms. In either case, the metabolic engineering approach of the present invention can be used to redirect carbon to oxaloacetate and, as a result, enhance protein and peptide yields.

Another alternative involves interfering with the metabolic pathway used to produce acetate from acetyl CoA. Disrupting this pathway should result in higher levels of acetyl CoA, which may then indirectly result in increased amounts of oxaloacetate. Moreover, where the pyruvate carboxylase enzyme that is expressed in the host cell is one that is activated by acetyl CoA, higher levels of acetyl CoA in these mutants leads to increased activity of the enzyme, causing additional carbon to flow from pyruvate to oxaloacetate. Thus, acetate$^-$ mutants are preferred host cells.

In one method, the metabolically engineered cell used in the invention is made by transforming a host cell with a nucleic acid fragment comprising a nucleotide sequence encoding a pyruvate carboxylase enzyme. Methods of transformation for bacteria, plant, and animal cells are well known in the art. Common bacterial transformation methods include electroporation and chemical modification. Transformation yields a metabolically engineered cell that overexpresses pyruvate carboxylase. Optionally, the cells are further transformed with a nucleic acid fragment comprising a nucleotide sequence encoding an enzyme having PEP carboxylase activity.

Preferably, the nucleic acid fragment is introduced into the cell using a vector, although "naked DNA" can also be used. The nucleic acid fragment can be circular or linear, single-stranded or double stranded, and can be DNA, RNA, or any modification or combination thereof. The vector can be a plasmid, a viral vector or a cosmid. Selection of a vector or plasmid backbone depends upon a variety of desired characteristics in the resulting construct, such as a selection marker, plasmid reproduction rate, and the like. Suitable plasmids for expression in *E. coli*, for example, include pUC(X), pKK223-3, pKK233-2, pTrc99A, and pET-(X) wherein (X) denotes a vector family in which numerous constructs are available. pUC(X) vectors can be obtained from Pharmacia Biotech (Piscataway, NH) or Sigma Chemical Co. (St. Louis, MO). pKK223-3, pKK233-2 and pTrc99A can be obtained from Pharmacia Biotech. pET-(X) vectors can be obtained from Promega (Madison, WI) Stratagene (La Jolla, CA) and Novagen (Madison, WI). To facilitate replication inside a host cell, the vector preferably includes an origin of replication (known as an "ori") or

replicon. For example, ColE1 and P15A replicons are commonly used in plasmids that are to be propagated in *E. coli*.

The nucleic acid fragment used to transform the cell according to the invention can optionally include a promoter sequence operably linked to the nucleotide sequence encoding the enzyme to be expressed in the host cell. A promoter is a DNA fragment which causes transcription of genetic material. Transcription is the formation of an RNA chain in accordance with the genetic information contained in the DNA. The invention is not limited by the use of any particular promoter, and a wide variety are known. Promoters act as regulatory signals that bind RNA polymerase in a cell to initiate transcription of a downstream (3' direction) coding sequence. A promoter is "operably linked" to a nucleic acid sequence if it is does, or can be used to, control or regulate transcription of that nucleic acid sequence. The promoter used in the invention can be a constitutive or an inducible promoter. It can be, but need not be, heterologous with respect to the host cell. Preferred promoters for bacterial transformation include *lac*, *lac*UV5, *tac*, *trc*, T7, SP6 and *ara*.

The nucleic acid fragment used to transform the host cell can, optionally, include a Shine Dalgarno site (e.g., a ribosome binding site) and a start site (e.g., the codon ATG) to initiate translation of the transcribed message to produce the enzyme. It can, also optionally, include a termination sequence to end translation. A termination sequence is typically a codon for which there exists no corresponding aminoacetyl-tRNA, thus ending polypeptide synthesis. The nucleic acid fragment used to transform the host cell can optionally further include a transcription termination sequence. The rrnB terminators, which is a stretch of DNA that contains two terminators, T1 and T2, is the most commonly used terminator that is incorporated into bacterial expression systems (J. Brosius et al., J. Mol. Biol., 148, 107-127 (1981)).

The nucleic acid fragment used to transform the host cell optionally includes one or more marker sequences, which typically encode a gene product, usually an enzyme, that inactivates or otherwise detects or is detected by a compound in the growth medium. For example, the inclusion of a marker sequence can render the transformed cell resistant to an antibiotic, or it can

confer compound-specific metabolism on the transformed cell. Examples of a marker sequence are sequences that confer resistance to kanamycin, ampicillin, chloramphenicol and tetracycline.

Pyruvate carboxylase can be expressed in the host cell from an expression vector containing a nucleic acid fragment comprising the nucleotide sequence encoding the pyruvate carboxylase. Alternatively, the nucleic acid fragment comprising the nucleotide sequence encoding pyruvate carboxylase can be integrated into the host's chromosome. Nucleic acid sequences, whether heterologous or endogenous with respect to the host cell, can be introduced into the cell's chromosome using, for example, homologous recombination. In bacteria, the gene of interest and a gene encoding a drug resistance marker are inserted into a piece of DNA on a plasmid that is homologous to the region of the chromosome within which the gene of interest is to be inserted. Next this recombinagenic DNA is introduced into the bacteria, and clones are selected in which the DNA fragment containing the gene of interest and drug resistant marker has recombined into the chromosome at the desired location. The gene and drug resistant marker can be introduced into the bacteria via transformation either as a linearized piece of DNA that has been prepared from any cloning vector, or as part of a specialized recombinant suicide vector that cannot replicate in the bacterial host, preferably a *recD*⁻ bacterial host. Clones are then verified using PCR and primers that amplify DNA across the region of insertion. PCR products from non-recombinant clones will be smaller in size and only contain the region of the chromosome where the insertion event was to take place, while PCR products from the recombinant clones will be larger in size and contain the region of the chromosome plus the inserted gene and drug resistance.

In another method, the metabolically engineered cell used in the invention is made by mutating the DNA of a cell that endogenously expresses a pyruvate carboxylase to alter transcription of the native pyruvate carboxylase gene so as to cause overproduction of the native enzyme. For example, a mutated chromosome can be obtained by employing either chemical or

transposon mutagenesis and then screening for mutants with enhanced pyruvate carboxylase activity using methods that are well-known in the art.

Optionally, the metabolically engineered cell used in the invention also overexpresses PEP carboxylase. In other words, the metabolically engineered cell optionally expresses PEP carboxylase at a level higher than the level of PEP carboxylase expressed in a comparable wild-type cell. As noted above, overproduction of PEP carboxylase alone has been shown to hamper glucose uptake in *E. coli*. However, overproduction of PEP carboxylase alone or in combination with overproduction of pyruvate carboxylase may nonetheless improve protein or peptide production in other cells, such as those that do not rely on PEP for glucose uptake. Additionally, in fermentations utilizing a carbon source other than glucose, such as fructose, overproduction of PEP carboxylase is not expected to negatively impact uptake of the alternative carbon source. Moreover, while overproduction of PEP carboxylase alone may adversely affect glucose uptake, it is possible that simultaneous overproduction of both pyruvate carboxylase and PEP carboxylase may not.

The levels of PEP carboxylase in different cell populations can be compared in any number of ways by one of skill in the art and is done under comparable growth conditions. For example, PEP carboxylase activity can be assayed, quantified and compared. In one assay, PEP carboxylase activity is measured in the absence of ATP using PEP instead of pyruvate as the substrate, by monitoring the appearance of CoA-dependent thionitrobenzoate formation at 412 nm (see Example II). The metabolically engineered cell that overexpresses PEP carboxylase will yield a greater PEP carboxylase activity than a wild-type cell. In addition, or alternatively, the amount of PEP carboxylase can be quantified and compared by preparing protein extracts from the cells, subjecting them to SDS-PAGE, transferring them to a Western blot, then detecting the PEP carboxylase protein using PEP antibodies in conjunction with detection kits available from Pierce Chemical Company (Rockford IL), Sigma Chemical Company (St. Louis, MO) or Boehringer Mannheim (Indianapolis, IN) for visualizing antigen-antibody complexes on Western blots. In a preferred

embodiment, the metabolically engineered cell expresses PEP carboxylase derived from a cyanobacterium, more preferably *Anacystis nidulans*.

The invention includes a method for making a protein or peptide by enhancing or augmenting production of the protein or peptide in a cell that is, prior to transformation as described herein, capable of biosynthesizing the protein or peptide. A metabolically engineered cell that overexpresses pyruvate carboxylase in accordance with the invention and which also produces a protein or peptide of interest is cultured to cause expression of the protein or peptide. Any type of cell culture or fermentation can be used, including but not limited to batch fermentations, fed-batch fermentations, continuous cultures, and perfusion cultures. The method of the invention allows enhanced production of the protein or peptide to be achieved, for example, by causing an increase in the protein activity or amount per cell, by causing an increase in the protein activity or amount per milliter of medium, by allowing cultures or fermentations to continue efficiently for longer periods of time, or through a combination of these effects. Protein or peptide production is enhanced compared to the level of production that can be achieved a comparable cell that does not overexpress pyruvate carboxylase. Advantageously, a higher yield of the protein or peptide is obtained in the metabolically engineered cell relative to similarly culturing a comparable host cell that does not overexpress pyruvate carboxylase. The term "yield" as used in connection with protein or peptide production in fermentations is usually expressed as a quotient of volumetric units, e.g., as units of activity per unit volume (typically milliliter) of media, divided by the change in substrate concentration during the fermentation, typically expressed in units of grams of substrate per liter of media. As an example, a fermentation of *E. coli* producing β-galactosidase could yield 2667 enzyme units (EU) of β-galactosidase (β-gal) per gram (g) of glucose consumed as follows:

$$\text{yield} = \frac{\text{80 EU of β-gal/mL of media}}{\text{30 g glucose/L of media}} = \text{2667 EU β-gal/g glucose}$$

Optionally, the method further includes metabolically engineering the host cell to overexpress pyruvate carboxylase, as described above, prior to culturing. Also optionally, the method further includes isolating the protein or peptide from the cultured cells. Proteins and peptides can be isolated from the cells using protocols, methods and techniques that are well-known in the art. Examples of protein isolation techniques include precipitations such as ammonium sulfate precipitation, filtration techniques, dialysis, phase extractions, chromatographic techniques including anion or cation exchange, hydroxyapatite, gel filtration, and affinity chromatography.

The invention further includes a method for increasing protein or peptide production in a host cell that, prior to applying the method of the invention, produces a given yield of protein or peptide of interest. The protein or peptide of interest can be a native or a recombinant protein, and the host cell can be a wild-type cell or a cell that has been genetically engineered. According to one method of the invention, the protein- or peptide-producing host cell is transformed with a nucleic acid fragment comprising a nucleotide sequence functionally encoding a pyruvate carboxylase enzyme to yield a metabolically engineered cell that produces a higher yield of the protein or peptide, compared with the host cell prior to transformation. Alternatively, the method involves mutating a pyruvate carboxylase gene of the host cell such that the host cell overexpresses pyruvate carboxylase to yield a metabolically engineered cell that produces a higher yield of the protein or peptide compared with the host cell prior to transformation. Protein- or peptide-producing host cells whose protein or peptide production can be enhanced in accordance with the method of the invention are as described in detail hereinabove and are not limited in any way to a particular type or class of cell.

The proteins and peptides that are produced or overproduced in, and isolated from, the metabolically engineered cells according to the method of the invention are not limited in any way and include native, mutant and recombinant polypeptides, including fusion proteins. They can be labeled with a detectable label, such as a radiolabel or fluorescent label, and can include known or unknown amino acid sequences and activities, and predetermined or randomized

amino acid sequences. While any desired polypeptide can be made according to the present invention, the invention is particularly well-suited to production of industrial enzymes, research and diagnostic enzymes, and therapeutic proteins and peptides. Industrial enzymes are those used in industrial processes or settings or in the production of consumer goods and services, such as amylase, glucoamylase, glucose isomerase, protease, lipase and cellulase. Research and diagnostic enzymes include those enzymes used in scientific or research settings such as restriction enzymes, for example *Hind* III, *EcoR* I and *BamH* I and DNA/RNA modifying enzymes, for example DNA or RNA polymerases, methylases, ligases, exonucleases, and kinases. Other proteins used in research include myoglobin, amidase, streptavidin ras protein, cholinesterase, and various human growth factors. Therapeutic proteins or protein drugs are those used for medical, nutritional or veterinary purposes and include, for example, erythropoietin, insulin, granulocyte colony stimulating factor, human growth hormone and interferon. Recombinant proteinaceous drugs that are currently produced in *E. coli* include aldesleukin (interleukin-2; IL-2), asparaginase, denileukin diftitox, filgrastim, growth hormone, insulin, interferon alfa-2a, interferon alfa-2b, interferon beta, interferon gamma-1b, oprelvekin (interleukin 11), and reteplase. Other recombinant proteinaceous drugs include alteplase, ancestim, basiliximab, becaplermin, coagulation factor VIIa, daclizumab, dornase alfa (recombinant human deoxyribonuclease; DNAse), epoetin alfa (erythropoietin; EPO), etanercept - a TNFα inhibitor, follitropins hepatitis B vaccine, imiglucerase, infliximab, lepirudin, lyme disease vaccine (recombinant OspA), palivizumab, rituximab, sargramostim (granulocyte macrophage colony stimulating factor; GM-CSF) and trastuzumab.

The invention further provides a novel protein expression system characterized by a protein-expressing or peptide-expressing cell that overexpresses pyruvate carboxylase. Any cellular protein expression system

capable of expressing a protein or peptide of interest can be modified in accordance with the invention by altering the protein- or peptide-producing cell to overexpress pyruvate carboxylase as described herein, to yield a protein expression system with enhanced protein or peptide yields.

EXAMPLES

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

Example I: Expression of the *R. etli* Pyruvate Carboxylase Enzyme Enables *E. coli* to Convert Pyruvate to Oxaloacetate

The *pyc* gene from *R. etli* was cloned into an *E. coli* expression vector and several experiments were conducted to determine whether active pyruvate carboxylase enzyme can be expressed in *E. coli*.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in LB Miller broth (rich) or M9 minimal media (J. Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1972)). Strains carrying a plasmid were supplemented with the appropriate antibiotic to detect the marker gene; ampicillin was used at 100 μg/ml in rich media and 50 μg/ml in minimal media while chloramphenicol was used at 20 μg/ml in rich media and 10 μg/ml in minimal media. When isopropyl β-D-thiogalactopyranoside (IPTG) was used to induce the pUC18-*pyc* construct, it was added at a final concentration of 1 mM.

TABLE 1: Strains and Plasmids

Strains	Genotype	Reference or source
MC1061	*ara*D139 (*ara*ABOIC-*leu*)7679 (*lac*)74 *gal*U *gal*K *rps*L *hsr hsm*+	M. Casadaban et al., J. Mol. Biol., 138, 179-207 (1980)
ALS225	MC1061 F'*lac*I^{q1}Z+Y+A+	E. Altman, University of Georgia
MG1665	wt	M. Guyer et al., Quant. Biol., Cold Spring Harbor Symp., 45, 135-140 (1980)
JCL 1242	(*arg*F-*lac*)U169 *ppc*::Kn	P. Chao et al., Appl. Env. Microbiol., 59, 4261-4265 (1993)

Plasmids	Relevant Characteristics	Reference or source
pUC18	Amp(R), ColE1 ori	J. Norrander et al., Gene., 26, 101-106 (1983)
pPC1	Tet(R), *pyc*	M. Dunn et al., J. Bacteriol., 178, 5960-5970 (1996)
pUC18-*pyc*	Amp(R), *pyc* regulated by P*lac*, ColE1 ori	This example
pBA11	Cam(R), *bir*A, P15A ori	D. Barker et al., J. Mol. Biol., 146, 469-492 (1981)

Construction of pUC18-*pyc*. The *R. etli pyc* gene, which encodes pyruvate carboxylase, was amplified using the polymerase chain reaction (PCR). *Pfu* polymerase (Stratagene, La Jolla, CA) was used instead of *Taq* polymerase and the pPC1 plasmid served as the DNA template. Primers were designed based on the published *pyc* gene sequence (M. Dunn et al., J. Bacteriol., 178, 5960-5970 (1996)) to convert the *pyc* translational start signals to match those of the *lac*Z gene. These primers also introduced a *Kpn*I (GGTACC) restriction site at the beginning of the amplified fragment and a *Bgl*II (AGATCT) restriction site at the end of the amplified fragment; forward primer 5' TAC TAT GGT ACC TTA GGA AAC AGC TAT GCC CAT ATC CAA GAT ACT CGT T 3' (SEQ ID NO:1), reverse primer 5' ATT CGT ACT CAG GAT CTG AAA GAT

CTA ACA GCC TGA CTT TAC ACA ATC G 3' (SEQ ID NO:2) (the *Kpn*I, Shine Dalgarno, ATG start, and *Bgl*II sites are underlined). The resulting 3.5 kb fragment was gel isolated, restricted with *Kpn*I and *Bgl*II and then ligated into gel isolated pUC18 DNA which had been restricted with *Kpn*I and *Bam*HI to form the pUC18-*pyc* construct. This construct, identified as "Plasmid in E. coli ALS225 pUC18-pyc", was deposited with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA, 20110-2209, USA, and assigned ATCC number 207111. The deposit was received by the ATCC on February 16, 1999.

Protein gels and Western blotting. Heat-denatured cell extracts were separated on 10% SDS-PAGE gels as per Altman et. al. (J. Bact., 155, 1130-1137 (1983)) and Western blots were carried out as per Carroll and Gherardini (Infect. Immun., 64, 392-398 (1996)). ALS225 *E. coli* cells containing either pUC18 or pUC18-*pyc* were grown to mid-log in rich media at 37°C both in the presence and absence of IPTG. Because ALS225 contains $lacI^{q1}$ on the F', significant induction of the pUC18-*pyc* construct should not occur unless IPTG is added. Protein extracts were prepared, subjected to SDS PAGE, and Western blotted. Proteins which had been biotinylated *in vivo* were then detected using the Sigma-Blot protein detection kit (Sigma Chemical Corp., St. Louis, MO). The instructions of the manufacturer were followed except that during the development of the western blots the protein biotinylation step was omitted, thus allowing for the detection of only those proteins which had been biotinylated *in vivo*.

Pyruvate carboxylase (PC) enzyme assay. For pyruvate carboxylase activity measurements, 100 mL of mid-log phase culture was harvested by centrifugation at 7,000 x g for 15 minutes at 4°C and washed with 10 mL of 100 mM Tris-Cl (pH 8.0). The cells were then resuspended in 4 mL of 100 mM Tris-Cl (pH 8.0) and subsequently subjected to cell disruption by sonication. The cell debris was removed by centrifugation at 20,000 x g for 15 minutes at 4°C. The pyruvate carboxylase activity was measured by the method of Payne and Morris (J. Gen. Microbiol., 59, 97-101 (1969)). In this assay the oxaloacetate produced by pyruvate carboxylase is converted to citrate by the

addition of citrate synthase in the presence of acetyl CoA and 5,5 -dithio-bis(2-nitro-benzoate) (DTNB) (Aldrich Chemical Co.); the homotetramer pyruvate carboxylase enzyme from *R. etli* requires acetyl coenzyme A for activation. The rate of increase in absorbence at 412 nm due to the presence of CoA-dependent formation of the 5-thio-2-nitrobenzoate was monitored, first after the addition of pyruvate and then after the addition of ATP. The difference between these two rates was taken as the ATP-dependent pyruvate carboxylase activity. The concentration of reaction components per milliliter of mixture was as follows: 100 mM Tris-Cl (pH 8.0), 5 mM $MgCl_2 \cdot H_2O$, 50 mM $NaHCO_3$, 0.1 mM acetyl CoA, 0.25 mM DTNB, and 5 units (U) of citrate synthase. Pyruvate, ATP, ADP, or aspartate, were added as specified in the Results section, below. The reaction was started by adding 50 µl of cell extract. One unit of pyruvate carboxylase activity corresponds to the formation of 1 µmol of 5-thio-2-nitrobenzoate per mg of protein per minute. All enzyme assays were performed in triplicate and a standard error of less then 10% was observed. The total protein in the cell extracts was determined by the Lowry method (O. Lowry et al., J. Biol. Chem., 193, 265-275 (1951)).

RESULTS

Expression of the *R. etli* pyruvate carboxylase enzyme in *E. coli*. The *R. etli pyc* gene, which encodes pyruvate carboxylase, was PCR amplified from pPC1 and subcloned into the pUC18 cloning/expression vector as described above. Because the translational start signals of the *R. etli pyc* gene were nonoptimal (*pyc* from *R. etli* uses the rare TTA start codon as well as a short spacing distance between the Shine Dalgarno and the start codon), the translational start signals were converted to match that of the *lacZ* gene which can be expressed at high levels in *E. coli* using a variety of expression vectors. When induced cell extracts of the pUC18-*pyc* construct were assayed via western blots developed to detect biotinylated proteins, a band of about 120 kD was detected. This value is consistent with the previously reported size assessment for the *R. etli* pyruvate carboxylase enzyme (M. Dunn et al., J.

Bacteriol., 178, 5960-5970 (1996)). By comparing serial dilutions of the pyruvate carboxylase which was expressed from the pUC18-*pyc* construct with purified pyruvate carboxylase enzyme obtained commercially, it was determined that, under fully induced conditions pyruvate carboxylase from *R. etli* was being expressed at 1% of total cellular protein in *E. coli*.

Effects of biotin and biotin holoenzyme synthase on the expression of biotinylated *R. etli* pyruvate carboxylase in *E. coli*. Pyruvate carboxylase is a biotin-dependent enzyme, and mediates the formation of oxaloacetate by a two-step carboxylation of pyruvate. In the first reaction step, biotin is carboxylated with ATP and bicarbonate as substrates, while in the second reaction the carboxyl group from carboxybiotin is transferred to pyruvate. All pyruvate carboxylases studied to date have been found to be biotin-dependent and exist as multimeric proteins, but the size and structure of the associated subunits can vary considerably. Pyruvate carboxylases from different bacteria have been shown to form α_4 or $\alpha_4\beta$ structures with the size of the α subunit ranging from 65 to 130 kD. In all cases, however, the α subunit of the pyruvate carboxylase enzyme has been shown to contain three catalytic domains - a biotin carboxylase domain, a transcarboxylase domain, and a biotin carboxyl carrier protein domain - which work collectively to catalyze the two-step conversion of pyruvate to oxaloacetate. In the first step, a biotin prosthetic group linked to a lysine residue is carboxylated with ATP and HCO_3^-, while in the second step, the carboxyl group is transferred to pyruvate. The biotinylation of pyruvate carboxylase occurs post-translationally and is catalyzed by the enzyme biotin holoenzyme synthase. In this experiment, *E. coli* cells containing the pUC18-*pyc* construct were grown under inducing conditions in minimal defined media which either contained no added biotin, or biotin added at 50 or 100 ng/mL. Specifically, MG1655 pUC18-*pyc* cells were grown to mid-log at 37°C in M9 media that contained varying amounts of biotin. Protein extracts were prepared, subjected to SDS PAGE, and Western blotted. Proteins which had been biotinylated *in vivo* were then detected using the Sigma-Blot protein detection kit, as described above. MG1655 was used in this experiment because it grows significantly faster than ALS225 in minimal media. Because MG1655 does not contain

lacI^{q1}, maximal expression of pyruvate carboxylase could be achieved without adding IPTG. The amount of biotinylated pyruvate carboxylase that was present in each sample was quantitated using a Stratagene Eagle Eye II Still Video. The biotinylation of pyruvate carboxylase that was expressed from the pUC18-*pyc* construct was clearly affected by biotin levels. Cells that had to produce all their biotin *de novo* expressed significantly lower amounts of biotinylated protein. The addition of biotin at a final concentration of 50 ng/mL was sufficient to biotinylate all of the pyruvate carboxylase that was expressed via the pUC18-*pyc* construct.

Since the post-translational biotinylation of pyruvate carboxylase is carried out by the enzyme biotin holoenzyme synthase, the effect of excess biotin holoenzyme synthase on the biotinylation of pyruvate carboxylase was investigated. This analysis was accomplished by introducing the multicopy plasmid pBA11 (which contains the *bir*A gene encoding biotin holoenzyme synthase) into *E. coli* cells that also harbored the pUC18-*pyc* construct; pBA11 is a pACYC184 derivative and thus compatible with pUC18-*pyc*. The effects of excess biotin holoenzyme synthase enzyme were examined in rich media where biotin would also be present in excess. Specifically, ALS225 cells containing pUC18-*pyc*, or pBA11 were grown to mid-log at 37°C in rich media that contained IPTG. Protein extracts were prepared, subjected to SDS PAGE, and Western blotted, and proteins which had been biotinylated *in vivo* were then detected using the Sigma-Blot protein detection kit as described above. Barker et al. (J. Mol. Biol., 146, 469-492 (1981)) have shown that pBA11 causes a 12-fold increase in biotin holoenzyme synthase enzyme levels. The amount of biotinylated pyruvate carboxylase that was present in each sample was quantitated using a Stratagene Eagle Eye II Still Video System. Protein extracts prepared from cells which either contained only pUC18-*pyc* or both pUC18-*pyc* and pBA11 yielded equal amounts of biotinylated pyruvate carboxylase protein. This result suggests that a single chromosomal copy of *bir*A is sufficient to biotinylate all of the pyruvate carboxylase that is expressed when biotin is present in excess.

R. etli pyruvate carboxylase can convert pyruvate to oxaloacetate in *E. coli*. To confirm that the expressed pyruvate carboxylase protein was enzymatically active in *E. coli*, the coupled enzyme assay developed by Payne and Morris was employed to assess pyruvate carboxylase activity (J. Payne et al., J. Gen. Microbiol., 59, 97-101 (1969)). Cell extracts containing the induced pUC18-*pyc* construct (MG1655 pUC18-*pyc*) were tested for pyruvate carboxylase activity using varying amounts of pyruvate, and compared to controls containing the pUC18 construct (MG1655 pUC18). ATP was added at a final concentration of 5 mM to the reaction mixture and pyruvate carboxylase activity was determined in the presence of increasing amounts of pyruvate. Fig. 2 shows that *E. coli* cells harboring the pUC18-*pyc* construct could indeed convert pyruvate to oxaloacetate and that the observed pyruvate carboxylase activity followed Michaelis-Menten kinetics. A Lineweaver-Burke plot of these data revealed that the saturation constant (K_m) for expressed pyruvate carboxylase was 0.249 mM with respect to pyruvate. This value is in excellent agreement with other pyruvate carboxylase enzymes that have been studied (H. Feir et al., Can. J. Biochem., 47, 698-710 (1969); H. Modak et al., Microbiol., 141, 2619-2628 (1995); M. Scrutton et al., Arch. Biochem. Biophys., 164, 641-654 (1974)).

It is well documented that the α4 pyruvate carboxylase enzymes can be inhibited by either aspartate or adenosine diphosphate (ADP). Aspartate is the first amino acid that is synthesized from oxaloacetate and ADP is liberated when pyruvate carboxylase converts pyruvate to oxaloacetate. Pyruvate carboxylase activity in the presence of each of these inhibitors was evaluated using extracts of MG1655 cells that contained the pUC18-*pyc* construct. The effect of aspartate was analyzed by adding ATP and pyruvate to the reaction mixture to final concentrations of 5 mM and 6 mM, respectively, then determining pyruvate carboxylase activity in the presence of increasing amounts of aspartate. Fig. 3 shows the pyruvate carboxylase activity that was obtained in the presence of different concentrations of aspartate. As expected, the pyruvate carboxylase activity was inhibited by aspartate and the specific activity decreased to approximately 43% in the presence of 8 mM aspartate. The effect of ADP was

analyzed by adding pyruvate to the reaction mixture to a final concentration of 5 mM, then determining pyruvate carboxylase activity in the presence of increasing amounts of ATP. Fig. 4 shows that ADP severely affected the observed pyruvate carboxylase activity and acted as a competitive inhibitor of ATP. A Lineweaver-Burke plot of these data revealed that the saturation constant (K_m) for expressed pyruvate carboxylase was 0.193 mM with respect to ATP and that the inhibition constant for ADP was 0.142 mM. Again, these values were in excellent agreement with other pyruvate carboxylase enzymes that have been studied H. Feir et al., Can. J. Biochem., 47, 698-710 (1969); H. Modak et al., Microbiol., 141, 2619-2628 (1995); M. Scrutton et al., Arch. Biochem. Biophys., 164, 641-654 (1974)).

To show that the expression of *R. etli* pyruvate carboxylase in *E. coli* can truly divert carbon flow from pyruvate to oxaloacetate, we tested whether the pUC18-*pyc* construct could enable an *E. coli* strain which contained a *ppc* null allele (*ppc* encodes PEP carboxylase) to grow on minimal glucose media. Because *E. coli* lacks pyruvate carboxylase and thus is only able to synthesize oxaloacetate from PEP, *E. coli* strains which contain a disrupted *ppc* gene can not grow on minimal media which utilizes glucose as the sole carbon source (P. Chao et al., Appl. Env. Microbiol., 59, 4261-4265 (1993)). The cell line used for this experiment was JCL1242 (*ppc*::kan), which contains a kanamycin resistant cassette that has been inserted into the *ppc* gene and thus does not express the PEP carboxylase enzyme. JCL1242 cells containing either pUC18 or the pUC18-*pyc* construct were patched onto minimal M9 glucose thiamine ampicillin IPTG plates and incubated at 37°C for 48 hours. As shown in Fig. 5, *E. coli* cells which contain both the *ppc* null allele and the pUC18-*pyc* construct were able to grow on minimal glucose plates. This complementation demonstrates that a branch point can be created at the level of pyruvate which results in the rerouting of carbon flow towards oxaloacetate, and clearly shows that pyruvate carboxylase is able to divert carbon flow from pyruvate to oxaloacetate in *E. coli*.

Example II. Expression of *R. etli* Pyruvate Carboxylase Causes Increased Succinate Production in *E. coli*

Experiments were conducted to investigate whether the overproduction of pyruvate carboxylase in *E. coli* could increase the production of an oxaloacetate-derived biochemical in an anaerobic fermentation. The end products which occur in an anaerobic fermentation are shown in Fig. 6. Succinate was chosen for this initial study because this biochemical is directly derived from oxaloacetate and can be easily measured.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* strains used in this study are listed in Table 2. The lactate dehydrogenase mutant strain designated RE02 was derived from MG1655 by P1 phage transduction using *E. coli* strain NZN111 (P. Bunch et al., Microbiol., 143, 187-195 (1997)).

TABLE 2: Strains and plasmids used.

Strains	Genotype	Reference or Source
MG1655	Wild type	M. Guyer et al., Quant. Biol., Cold Spring Harbor Symp., 45, 135-140 (1980)
RE02	MG1655 *ldh*	This example

Plasmids	Relevant Characteristics	Reference or Source
pUC18-*pyc*	Amp(R), *pyc* regulated by P*lac*	Example I
pTrc99A	Amp(R), lacI^q, P*trc*	E. Amann et al., Gene, 69:301-315 (1988)
pTrc99A-*pyc*	Amp(R), lacI^q, *pyc* regulated by P*trc*	This example

The *pyc* gene from *R. etli* was originally cloned under the control of the *lac* promoter (Example I). Because this promoter is subjected to catabolic repression in the presence of glucose, a 3.5 kb *Xba*I-*Kpn*I fragment from pUC18-*pyc* was ligated into the pTrc99A expression vector which had been digested with *Xba*I and *Kpn*I. The new plasmid was designated as pTrc99A-*pyc*. This plasmid, identified as "Plasmid in E. coli ALS225 pTrc99A-pyc", was deposited with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA, 20110-2209, USA, and assigned ATCC number 207112. The deposit was received by the ATCC on February 16, 1999. In this new construct the transcription of the *pyc* gene is under the control of artificial *trc* promoter and thus is not subjected to catabolic repression in the presence of glucose.

Media and growth conditions. For strain construction, *E. coli* strains were grown aerobically in Luria-Bertani (LB) medium. Anaerobic fermentations were carried out in 100 mL serum bottles with 50 mL LB medium supplemented with 20 g/L glucose and 40 g/L $MgCO_3$. The fermentations were terminated at 24 hours at which point the pH values of all fermentations were approximately pH 6.7, and glucose was completely utilized. For plasmid-containing strains either ampicillin or carbenicillin was added to introduce selective pressure during the fermentation. Each of these antibiotics was introduced initially at 100 μg/mL. In one set of experiments, no additional antibiotic was added during fermentation, while in a second set of experiments an additional 50 μg/mL was added at 7 hours and 14 hours. Pyruvate carboxylase was induced by adding 1 mM IPTG. For enzyme assays cells were grown in LB medium supplemented with 20 g/L glucose and buffered with 3.2 g/L Na_2CO_3.

Fermentation product analysis and enzyme assays. Glucose, succinate, acetate, formate, lactate, pyruvate and ethanol were analyzed by high-pressure liquid chromatography (HPLC) using a Coregel 64-H ion-exclusion column (Interactive Chromatography, San Jose, CA) and a differential refractive index

detector (Model 410, Waters, Milford, MA). The eluant was 4 mN H_2SO_4 and the column was maintained at 60°C.

For enzyme activity measurements, 50 mL of mid-log phase culture were harvested by centrifugation (10000 x g for 10 minutes at 4°C) and washed with 10 mL of 100 mM Tris-HCl buffer (pH 8.0). The cells were then resuspended in 2 mL of 100 mM Tris-HCl buffer and subjected to cell disruption by sonication. Cell debris were removed by centrifugation (20000 x g for 15 minutes at 4°C Pyruvate carboxylase activity (J. Payne et al., J. Gen. Microbiol. 59, 97-101 (1969); see also Example I), and the endogenous activities of PEP carboxylase (K. Terada et al., J. Biochem., 109, 49-54 (1991)), malate dehydrogenase and lactate dehydrogenase (P. Bunch et al., Microbiol., 143, 187-195 (1997)) were then measured. The total protein in the cell extract was determined using the Lowry method.

RESULTS

Table 3 shows that pyruvate carboxylase activity could be detected when the pTrc99A-*pyc* construct was introduced into either wild type cells (MG1655) or wild type cells which contained a *ldh*$^-$ null mutation (RE02). The presence of IPTG did not significantly affect the expression of other important metabolic enzymes such as PEP carboxylase, lactate dehydrogenase and malate dehydrogenase.

TABLE 3: Enzyme activity in exponential phase cultures.

		Specific Activity (μmol/min mg protein)			
Strain	IPTG	Pyruvate carboxylase	PEP carboxylase	Lactate dehydrogenase	Malate dehydrogenase
MG1655	-	0.00	0.15	0.31	0.06
	+	0.00	0.18	0.38	0.06
MG1655 pTrc99A-*pyc*	-	0.00	0.15	0.32	0.05
	+	0.22	0.11	0.32	0.05
RE02	-	0.00	0.15	0.00	0.04
	+	0.00	0.13	0.00	0.04
RE02 pTrc99A-*pyc*	-	0.00	0.15	0.00	0.04
	+	0.32	0.12	0.00	0.05

TABLE 4: Effect of pyruvate carboxylase on product distribution from *E. coli* glucose fermentation.

Strain	Antibiotic	Mode of antibiotic addition_	Pyruvate (g/L)	Succinate (g/L)	Lactate (g/L)	Formate (g/L)	Acetate (g/L)	Ethanol (g/L)
MG1655 (wt)	-	-	0.00 (0.00)	1.57 (0.17)	4.30(0.73)	4.34 (0.50)	3.34 (0.36)	2.43 (0.24)
MG1655 pTrc99A-*pyc*	Amp	1x	0.00 (0.00)	4.36 (0.45)	2.22 (0.49)	3.05 (0.57)	3.51 (0.03)	2.27 (0.30)
MG1655 pTrc99A-*pyc*	Car	1x	0.00 (0.00)	4.42(0.44)	2.38 (0.76)	2.94 (0.46)	3.11 (0.36)	2.27(0.36)
MG1655pTrc99A-*pyc*	Amp	3x	0.00 (0.00)	4.41 (0.07)	1.65(0.08)	4.17 (0.15)	3.93 (0.11)	2.91 (0.34)
MG1655 pTrc99A-*pyc*	Car	3x	0.00 (0.00)	4.37 (0.06)	1.84 (0.07)	4.09 (0.08)	3.88 (0.06)	2.58 (0.09)
RE02 (*ldh*-)	-	-	0.61 (0.06)	1.73 (0.12)	0.00 (0.00)	6.37 (0.46)	4.12 (0.30)	3.10 (0.26)
RE02 pTrc99A-*pyc*	Amp	1x	0.33 (0.11)	2.92 (0.12)	0.00 (0.00)	5.38 (0.12)	4.09 (0.16)	2.53 (0.03)
RE02 pTrc99A-*pyc*	Car	1x	0.25 (0.05)	2.99 (0.55)	0.00 (0.00)	5.50 (0.90)	4.23 (0.71)	2.50 (0.44)
RE02pTrc99A-*pyc*	Amp	3x	0.30 (0.04)	2.74 (0.07)	0.00 (0.00)	6.48 (0.04)	4.75(0.06)	2.99 (0.03)
RE02 pTrc99A-*pyc*	Car	3x	0.33 (0.04)	2.65 (0.05)	0.00 (0.00)	6.21 (0.18)	4.60(0.12)	3.05 (0.07)

In order to elucidate the effect of pyruvate carboxylase expression on the distribution of the fermentation end products, several 50 mL serum bottle fermentations were conducted (see Table 4).

Antibiotics either added once at 0 hours at a concentration of 100 μg/mL (1x) or added at 0 hours at a concentration of 100 μg/mL and again at 7 hours and 14 hours at 50 μg/mL (3x). Values are the mean of three replicates and standard deviations are shown in parentheses. To calculate the net yield of each product per gram of glucose consumed, the final product concentration is divided by 20g/L of glucose.

As shown in Table 4, expression of pyruvate carboxylase caused a significant increase in succinate production in both MG1655 (wild type) and RE02 (*ldh*⁻). With MG1655 the induction of pyruvate carboxylase increased the production of succinate 2.7-fold from 1.57 g/L in the control strain to 4.36 g/L, thus making succinate the major product of glucose fermentation. This increase in succinate was accompanied by decreased lactate and formate formation, indicating that carbon was diverted away from lactate toward succinate formation. A similar carbon diversion from lactate toward succinate was achieved previously by the overexpression of native PEP carboxylase (C. Millard et al., Appl. Environ. Microbiol., 62, 1808-1810 (1996)). Table 4 also shows that ampicillin and carbenicillin were equally effective in maintaining sufficient selective pressure, and that the addition of more of either antibiotic during the fermentation did not further enhance the succinate production. This evidence indicates that an initial dose (of 100 μg/mL) is sufficient to maintain selective pressure throughout the fermentation, a result which might be due to the relatively high final pH (6.8) observed in our fermentation studies versus the final pH (6.0) observed in previous studies (C. Millard et al., Appl. Environ. Microbiol., 62, 1808-1810 (1996)).

Because introducing pyruvate carboxylase into *E. coli* was so successful at directing more carbon to the succinate branch, we were also interested in determining whether additional carbon could be directed to succinate by eliminating lactate dehydrogenase, since this enzyme also competes for pyruvate. Table 4 compares the results of fermentations using the RE02 (*ldh*⁻) strain with or without the pTrc99A-*pyc* plasmid. Compared to the wild type strain (MG1655), the

RE02 strain showed no significant change in succinate production. Instead, fermentations with the RE02 strain, whether it contained the pTrc99A-*pyc* plasmid or not, resulted in increased formate, acetate and ethanol production, accompanied by secretion of pyruvate. The fact that pyruvate was secreted into the fermentation broth indicates that the rate of glycolysis was greater than the rate of pyruvate utilization. The observed increase in formate concentrations in the *ldh*⁻ mutant may be caused by the accumulation of pyruvate, a compound which is known to exert a positive allosteric effect on pyruvate formate lyase (G. Sawers et al., J. Bacteriol., 170, 5330-5336 (1988)). With RE02 the induction of pyruvate carboxylase increased the production of succinate 1.7-fold from 1.73 g/L in the control strain to 2.92 g/L. Thus, the succinate increase obtained in the *ldh*⁻ mutant strains was significantly lower than that obtained in the wild type strain (MG1655). A possible explanation for this observation might be that pyruvate carboxylase activity was inhibited by a cellular compound which accumulated in the *ldh*⁻ mutants.

During glycolysis two moles of reduced nicotinamide adenine dinucleotide (NADH) are generated per mole of glucose. NADH is then oxidized during the formation of ethanol, lactate and succinate under anaerobic conditions. The inability of the *ldh*⁻ mutants to consume NADH through lactate formation may put stress on the oxidizing capacity of these strains, leading to an accumulation of NADH. Indeed, this reduced cofactor has previously been shown to inhibit a pyruvate carboxylase isolated from *Saccharomyces cerevisiae* (J. Cazzulo et al., Biochem. J., 112, 755-762 (1969)). In order to elucidate whether such oxidizing stress might be the cause of the attenuated benefit that was observed when pyruvate carboxylase was expressed in the *ldh*⁻ mutants, we investigated the effect of both oxidized and reduced nicotinamide adenine dinucleotide ($NADH/NAD^+$) and dinucleotide phosphate ($NADPH/NADP^+$) on pyruvate carboxylase activity. Enzyme assays were conducted with cell-free crude extract obtained from MG1655 pTrc99A-*pyc*. All assays were conducted in triplicate, and average values are shown in Fig. 7. Standard deviation was no greater than 5% for all data points. NADH inhibited pyruvate carboxylase, whereas NAD^+, $NADP^+$ and NADPH did not. The lower succinate enhancement with RE02 the *ldh*⁻ mutant is therefore

hypothesized to result from an accumulation of intracellular NADH, a cofactor which appears to inhibit pyruvate carboxylase activity.

Example III. Expression of *R. etli* Pyruvate Carboxylase Does Not Affect Glucose Uptake in *E. coli* in Anaerobic Fermentation

Experiments were conducted to test whether the overproduction of pyruvate carboxylase in *E. coli* adversely affected glucose uptake.

METHODS

Microorganisms and plasmids. *E. coli* strain MG1655 (wild type F λ ; M. Guyer et al., Quant. Biol., Cold Spring Harbor Symp., 45, 135-140 (1980); see also Example I) and the plasmid pUC18-*pyc* which contains the *pyc* gene from *R. etli* (see Example I).

Media and fermentation. All 2.0 L fermentations were carried out in 2.5 L New Brunswick BioFlo III bench top fermenters (New Brunswick Scientific, Edison, NJ) in Luria-Bertani (LB) supplemented with glucose, 10 g/L; $Na_2PHO_4 \cdot 7H_2O$, 3 g/L; KH_2PO_4, 1.5 g/L; NH_4Cl, 1 g/L; $MgSO_4 \cdot 7H_2O$, 0.25 g/L; and $CaCl_2 \cdot 2H_2O$, 0.02 g/L. The fermenters were inoculated with 50 mL of anaerobically grown culture. The fermenters were operated at 150 rpm, 0% oxygen saturation (Ingold polarographic oxygen sensor, New Brunswick Scientific, Edison, NJ), 37°C, and pH 6.4, which was controlled with 10% NaOH. Anaerobic conditions were maintained by flushing the headspace of the fermenter with oxygen-free carbon dioxide. When necessary, the media was supplemented with an initial concentration of 100 µg/mL ampicillin, previously shown to be sufficient to maintain the selective pressure (Example II).

Analytical methods. Cell growth was monitored by measuring the optical density (OD) (DU-650 spectrophotometer, Beckman Instruments, San Jose, CA) at 600 nm. This optical density was correlated with dry cell mass using a calibration curve of dry cell mass (g/L) = 0.48 OD. Glucose and fermentation products were analyzed by high-pressure liquid chromatography

using Coregel 64-H ion-exclusion column (Interactive Chromatography, San Jose, CA) as described in Example II.

The activity of pyruvate carboxylase and the endogenous activity of PEP carboxylase was measured by growing each strain and clone separately in 160 mL serum bottles under strict anaerobic conditions. Cultures were harvested in mid-logarithmic growth, washed and subjected to cell disruption by sonication. Cell debris were removed by centrifugation (20000 g for 15 minutes at 4°C). Pyruvate carboxylase activity was measured as previously described (Payne and Morris, 1969), and the PEP carboxylase activity was measured in the absence of ATP using PEP instead of pyruvate as the substrate, with the appearance of CoA-dependent thionitrobenzoate formation at 412 nm monitored. The total protein in the cell extract was determined using the Lowry method.

RESULTS

E. coli MG1655 was grown anaerobically with 10 g/L glucose as energy and carbon source. Fig. 8 shows the dry cell mass, succinate, lactate, formate and glucose concentrations with time in a typical 2-liter fermentation of this wild-type strain. Fig. 9 shows these concentrations with time in a fermentation of this wild-type strain with the cloning/expression vector pUC18. After complete glucose utilization, the average final concentration of succinate for the wild-type strain was 1.18 g/L, while for the wild-type strain with the vector pUC18 the final succinate concentration was 1.00 g/L. For these fermentations, the average final lactate concentration was 2.33 g/L for the wild-type strain and 2.27 g/L for the same strain with pUC18.

Fig. 10 shows the concentrations with time of dry cell mass, succinate, lactate, formate and glucose in a fermentation of the strain containing the pUC18-*pyc* plasmid. This figure shows that the expression of pyruvate carboxylase causes a substantial increase in final succinate concentration and a decrease in lactate concentration. Specifically, for the wild-type with pUC18-*pyc* the average final succinate concentration was 1.77 g/L, while the average final lactate concentration was 1.88 g/L. These concentrations correspond to a

50% increase in succinate and about a 20% decrease in lactate concentration, indicating that carbon was diverted from lactate toward succinate formation in the presence of the pyruvate carboxylase.

The activities of PEP carboxylase and pyruvate carboxylase were assayed in cell-free extracts of the wild type and the plasmid-containing strains, and these results are shown in Table 5. In the wild type strain and the strain carrying the vector no pyruvate carboxylase activity was detected, while this activity was detected in MG1655/pUC18-*pyc* clone. PEP carboxylase activity was observed in all three strains.

TABLE 5. Enzyme activity in mid-logarithmic growth culture.

	Sp. activity (μmol/min mg protein)	
Strain	Pyruvate carboxylase	PEP carboxylase
MG1655	0.0	0.10
MG1655/pUC18	0.0	0.12
MG1655/pUC18-*pyc*	0.06	0.08

To determine the rates of glucose consumption, succinate production, and cell mass production during the fermentations, each set of concentration data was regressed to a fifth-order polynomial. (These best-fitting curves are shown in Figs. 8-10 with the measured concentrations.) By taking the first derivative of this function with respect to time, an equation results which provides these rates as functions of time. This procedure is analogous to previous methods (E. Papoutsakis et al., Biotechnol. Bioeng, 27, 56B66 (1985); K. Reardon et al., Biotechnol. Prog, 3, 153B167 (1987)) used to calculate metabolic fluxes. In the case of fermentations with both pyruvate carboxylase and PEP carboxylase

present, however, the flux analysis cannot be completed due to a mathematical singularity at the PEP/pyruvate nodes (S. Park et al., Biotechnol. Bioeng, 55, 864B879 (1997)). Nevertheless, using this approach the glucose uptake and the rates of succinate and cell mass production may be determined.

Table 6 shows the results of calculating the rates of glucose uptake, and succinate and cell mass production in a wild-type *E. coli* strain (MG1655), the wild-type strain with the pUC18 cloning/expression vector (MG1655/pUC18) and the wild-type strain with MG1655/pUC18-*pyc*. All units are g/Lh, and the values in parentheses represent standard deviation of measurements.

TABLE 6. Rates of glucose uptake, succinate production, and cell production.

Parameter	MG1655	MG1655/pUC18	MG1655/pUC18-*pyc*
Glucose uptake (maximum)	2.17 (0.10)	2.40 (0.01)	2.47 (0.01)
Glucose uptake (average during final 4 h of fermentations)	1.99 (0.05)	2.00 (0.06)	1.99 (0.05)
Rate of succinate production (at time of max. glucose uptake)	0.234 (0.010)	0.200 (0.012)	0.426 (0.015)
Rate of succinate production (average during final 4 h)	0.207 (0.005)	0.177 (0.009)	0.347 (0.002)
Cell production (maximum)	0.213 (0.006)	0.169 (0.033)	0.199 (0.000)

As these results demonstrate, the addition of the cloning vector or the vector with the *pyc* gene had no significant effect on the average glucose uptake during the final 4 hours of the fermentations. Indeed, the presence of the *pyc* gene actually increased the maximum glucose uptake about 14% from 2.17 g/Lh to 2.47 g/Lh. The presence of the pUC18 cloning vector reduced slightly the rates of succinate production. As expected from the data shown in Fig. 10, the expression of the *pyc* gene resulted in an 82% increase in succinate production at the time of maximum glucose uptake, and a 68% increase in the rate of succinate production during the final 4 hours of the fermentations. The maximum rate of cell growth (which occurred at 4-5 hours for each of the fermentations) was 0.213 g/Lh in the wild type strain, but decreased in the presence of pUC18 (0.169 g/Lh) or pUC18-*pyc* (0.199 g/Lh). Similarly, the overall cell yield was 0.0946 g dry cells/g glucose consumed for the wild-type, but 0.0895 g/g for the wild-type with pUC18 and 0.0882 g/g for the wild-type strain with pUC18-*pyc*. This decrease in biomass may be due to the expenditure of one mole of energy unit (ATP) per mole of pyruvate converted to oxaloacetate by pyruvate carboxylase and the increased demands of protein synthesis in the plasmid-containing strains. A specific cell growth rate could not be calculated since the growth of this strain shows logarithmic growth only for the first few hours of growth.

In summary, expression of pyruvate carboxylase from *R. etli* in *E. coli* causes a significant increase in succinate production without affecting glucose uptake. This result has dramatic ramifications for bacterial fermentation processes which are used to produce oxaloacetate-derived biochemicals. Because overexpression of pyruvate carboxylase causes increased production of oxaloacetate-derived biochemicals without affecting glucose uptake, this technology can be advantageously employed in fermentation processes in order to obtain more product per amount of inputted glucose.

Example IV. Expression of *R. etli* Pyruvate Carboxylase Causes Increased Threonine Production in *E. coli*

Experiments were conducted to investigate whether the overproduction of pyruvate carboxylase in *E. coli* during an aerobic fermentation could increase the production of an amino acid that is derived from oxaloacetate.

MATERIALS AND METHODS

Bacterial strains and plasmids. The threonine-producing strain βIM-4 (ATCC 21277) was used in this study (I. Shiio et al., Agr. Biol. Chem., 33, 1152-1160 (1969); I. Shiio et al. U.S. Pat. No., 3,580,810 (1971)). This strain was transformed with either pTrc99A-*pyc* (see Example II) or pTrc99A (E. Amann et al., Gene, 69, 301-315 (1988)).

Media and growth conditions. Aerobic fermentations were carried out in 2.0 L volume in Bioflow II Fermenters. The media used for these fermentation contained (per liter): glucose, 30.0 g; $(NH_4)_2SO_4$ 10.0 g, $FeSO_4 \cdot H_2O$, 10.0 mg; $MnSO_4 \cdot H_2O$, 5.5 mg/L; L-proline, 300 mg; L-isoleucine, 100 mg; L-methionine, 100 mg; $MgSO_4 \cdot 7H_2O$, 1 g; KH_2PO_4, 1 g; $CaCO_3$, 20 g; thiamine·HCl, 1mg; d-biotin, 1 mg. In order to maintain selective pressure for the plasmid-carrying strains, media were supplemented initially with 50 mg/L ampicillin. Also, IPTG was added to a final concentration of 1 mmol/L at 2 hours to fermentations performed with either of these strains.

Fermentation product analysis. Cell growth was determined by measuring optical density at 550 nm of a 1:21 dilution of sample in 0.1M HCl. Glucose, acetic acid and other organic acids were analyzed by high-pressure liquid chromatography as previously described (M. Eiteman et al., Anal. Chim. Acta, 338, 69-75 (1997)) using a Coregel 64-H ion-exclusion column. Threonine was quantified by high-pressure liquid chromatography using the ortho-phthaldialdehyde derivatization method (D. Hill et al., Anal. Chem., 51, 1338-1341 (1979); V. Svedas et al. Anal. Biochem., 101, 188-195 (1980)).

RESULTS

The threonine-producing strain βIM-4 (ATCC 21277), harboring either the control plasmid pTrc99A or the plasmid pTrc99A-*pyc* which overproduces pyruvate carboxylase, was grown aerobically with 30 g/L glucose as energy and carbon source and the production of threonine was measured. As shown in Fig. 11, the overproduction of pyruvate carboxylase caused a significant increase in the production of threonine in the threonine-producing *E. coli* strain. At 17 hours when the initial inputted glucose had been consumed, a concentration of 0.57 g/L threonine was detected in the parental strain harboring the pTrc99A control plasmid, while a concentration of 1.37 g/L threonine was detected in the parental strain harboring the pTrc99A-*pyc* plasmid. Given that the final OD_{550} of both cultures were within 10% of each other at the end of the fermentation, the 240% increase in threonine concentration caused by the overproduction of pyruvate carboxylase can be deemed to be significant. As in our anaerobic fermentation studies (see Example III), we found that glucose uptake was not adversely affected by the overproduction of pyruvate carboxylase.

Example V. Enhancement of Biochemical Production Using Pyruvate Carboxylase from *P. fluorescens*

One of the main reasons the metabolic network responsible for regulating the intracellular levels of oxaloacetate is so tightly controlled is due to the fact that the key enzymes which are involved in this process are both positively and negatively regulated. In most organisms such as *R. etli*, pyruvate carboxylase requires the positive effector molecule acetyl coenzyme A for its activation and is repressed due to feedback inhibition by aspartate (P. Attwood, Intl. J. Biochem. Cell Biol., 27, 231-249 (1995); M. Dunn et al., J. Bacteriol., 178, 5960-5970 (1996)). The benefits obtained from overproducing *R. etli* pyruvate carboxylase are thus limited by the fact that diverting carbon from pyruvate to oxaloacetate both depletes acetyl coenzyme A levels and increases aspartate levels. The pyruvate carboxylase from *P. fluorescens*, however, does not require acetyl coenzyme A for its activation and it is not affected by the feedback

inhibition caused by aspartate (R. Silvia et al., J. Gen. Microbiol., 93, 75-81 (1976)). Overproduced *P. fluorescens* pyruvate carboxylase should allow even more carbon flow to be diverted towards oxaloacetate.

Because the genes encoding pyruvate carboxylases in bacteria appear to be highly homologous, the *P. fluorescens pyc* gene may be readily isolated from a genomic library using probes which have been prepared from the *R. etli* gene. The gene for pyruvate carboxylase in *P. fluorescens* will thus be identified, isolated, and cloned into an expression vector using standard genetic engineering techniques. Alternatively, the pyruvate carboxylase enzyme can be isolated and purified from *P. fluorescens* by following pyruvate carboxylase activity (as described in the above Examples) and also by assaying for biotinylated protein using Western blots. The N-terminal amino acid sequence of the purified protein is determined, then a degenerate oligonucleotide probe is made which is used to isolate the gene encoding pyruvate carboxylase from a genomic library that has been prepared from *P. fluorescens*. The *pyc* clone thus obtained is sequenced. From the sequence data, oligonucleotide primers are designed that allow cloning of this gene into an expression vector so that pyruvate carboxylase can be overproduced in the host cell. Either method can be used to yield a vector encoding the *P. fluorescens pyc* gene, which is then used to transform the host *E. coli* or *C. glutamicum* cell. Pyruvate carboxylase from *P. fluorescens* is expressed in the host cell, and biochemical production is enhanced as described in the preceding examples.

Example VI. Enhancement of Biochemical Production By Overexpression of Both Pyruvate Carboxylase and PEP Carboxylase

In many organisms PEP can be carboxylated to oxaloacetate via PEP carboxylase or it can be converted to pyruvate by pyruvate kinase (I. Shiio et al., J. Biochem., 48, 110-120 (1960); M. Jetten et al., Appl. Microbiol. Biotechnol., 41, 47-52 (1994)). One possible strategy that was tried to increase the carbon flux toward oxaloacetate in *C. glutamicum* was to block the carbon flux from PEP toward pyruvate. However, lysine production by pyruvate kinase mutants

was 40% lower than by a parent strain, indicating that pyruvate is essential for high-level lysine production (M. Gubler et al., Appl. Microbiol. Biotechnol., 60, 47-52 (1994)).

Carbon flux toward oxaloacetate may be increased by overexpressing PEP carboxylase in conjunction with overexpressed pyruvate carboxylase without concomitantly blocking carbon flux from PEP to pyruvate or affecting glucose uptake.

In heterotrophs such as *C. glutamicum*, however, PEP carboxylase requires acetyl-CoA for its activation, and is inhibited by aspartate (S. Mike et al., Annals NY Acad. Sci., 272, 12-29 (1993)); hence amplification of *C. glutamicum* PEP carboxylase genes has not resulted in increased lysine yield (J. Kremer et al., Appl. Environ. Microbiol., 57, 1746-1752 (1991)). PEP carboxylase isolated from the cyanobacteria *Anacystis nidulans*, however, does not require acetyl CoA for activation nor is it inhibited by aspartate (M. Utter et al., Enzymes, 6, 117-135 (1972)). Therefore, this heterologous enzyme can be used to increase the carbon flux towards oxaloacetate in *C. glutamicum*. The genes encoding PEP carboxylase in *A. nidulans* have been isolated and cloned (T. Kodaki et al., J. Biochem., 97, 533-539 (1985)).

Example VII. Enhancement of Biochemical Production By Disrupting the *pck* Gene Encoding PEP Carboxykinase in Conjunction with Overexpressed Pyruvate Carboxylase

Some of carbon which is diverted to oxaloacetate via overproduced pyruvate carboxylase is likely converted back to PEP due to the presence of PEP carboxykinase. More carbon can be diverted towards oxaloacetate in these systems if the host cell contains a disrupted *pck* gene, such as an *E. coli* strain which contains a *pck* null allele (e.g., A. Goldie, J. Bacteriol., 141, 1115-1121 (1980)).

Example VIII. Enhanced Yield of Recombinant Proteins in *E. coli* Expressing Pyruvate Carboxylase

To determine the effect that pyruvate carboxylase had on the production of recombinant proteins, several experiments were conducted using *E. coli* strains which either overproduced the native β-galactosidase enzyme or the non-native catechol 2,3-dioxygenase enzyme.

MATERIALS AND METHODS

Bacterial strains and plasmids. All cloning experiments were done in the R^-M^+ *E. coli* strain ALS226 which is MC1061 / F'*lacI*^{Q1} *Z*::Tn5 *Y*$^+$ *A*$^+$ (E. Altman, University of Georgia). The wild-type *E. coli* strain MG1655, F- λ- (M. Guyer et al., Cold Spring Harbor Symp. Quant. Biol. 45: 135-140 (1980)), was used for all of the other studies. The plasmids used in this work are described in Table 7.

Table 7. Plasmids

Plasmid Name	Relevant Characteristics	Reference or Source
pACYC184	Cam^R, Tet^R, P15A replicon	A. Chang et al., J. Bacteriol. 134: 1141-1156 (1978)
pTer7	wild-type *lacZ* coding region, Amp^R	R. Young, Texas A&M University
pTrc99A	*trc* promoter / operator, $lacI^Q$, Amp^R, ColE1 replicon	E. Amann et al., Gene 69: 301-315 (1988)
pTrc99A-*pyc*	*trc* promoter / operator, *Rhizobium etli pyc* gene, $lacI^Q$, Amp^R, ColE1 replicon (*pyc* in pTrc99A)	Example II
pXE60	wild-type TOL pWWO *xylE* gene, Amp^R	J. Westpheling, University of Georgia

Construction of pACYC184-*lacZ* and pACYC184-*xylE*. Initially the *lacZ* and *xylE* genes were cloned into the pTrc99A expression vector. To construct pTrc99A-*lacZ*, primers 5' TAT CAT GGA TCC AGG AAA CAG CTA TGA CCA TGA TTA CGG ATT CAC TG 3' (SEQ ID NO:3) and 5' TAC ATA CTC GAG CAG GAA AGC TTG GCC TGC CCG GTT ATT ATT ATT TT 3' (SEQ ID NO:4) were used to PCR amplify a 3138 base pair (bp) fragment from the pTer7 plasmid (restriction enzyme sites are indicated with a double underline while the regions of homology to *lacZ* are indicated by with a single underline). The resulting fragment was gel isolated, digested with *Bam*H I and *Hind* III, and then ligated into the pTrc99A vector which had been digested with the same two restriction enzymes. To construct pTrc99A-*xylE*, primers 5' ATC AGA CTG CAG GAG GTA ACA GCT ATG AAC AAA GGT GTA ATG CGA CC 3' (SEQ ID NO:5) and 5' TAG CAG TGG CAG CTC TGA AAG CTT TGC ACA ATC TCT GCA ATA AGT CG 3' (SEQ ID NO:6) were used to PCR amplify a 1006 bp fragment from the pXE60 plasmid which contains the wild-type *Pseudomonas putida xylE* gene isolated from the TOL pWWO plasmid (restriction enzyme sites are indicated with a double underline while the regions of homology to *xylE* are indicated by with a single underline). The resulting fragment was gel isolated, digested with *Pst* I and *Hind* III, and then ligated into the pTrc99A vector which had been digested with the same two restriction enzymes.

To construct pACYC184-*lacZ*, a 3504 bp fragment from pTrc99A-*lacZ* that contained the *lacZ* gene and the *trc* promoter was ligated to a 3178 bp fragment from pACYC184 that contained the *cat* gene and the ori of replication. The 3054 bp *lacZ* fragment was prepared by digesting pTrc99A-*lacZ* with *Nar* I and *Hind* III and then filling in with Klenow, while the 3178 bp fragment containing *cat* and the ori was prepared by digesting pACYC184 with *Hind* III. A clone was then selected which transcribed *lacZ* in the clockwise direction with respect to pACYC184. To construct pACYC184-*xylE*, a 1365 bp fragment from pTrc99A-*xylE* that contained the *xylE* gene and the *trc* promoter was ligated to a 3178 bp fragment from pACYC184 that contained the *cat* gene and the ori of replication. The 1365 bp

xylE fragment was prepared by digesting pTrc99A-*xylE* with *Nar* I and *Hind* III and then filling in with Klenow, while the 3178 bp fragment containing *cat* and the ori was prepared by digesting pACYC184 with *Hind* III. A clone was then selected which transcribed *xylE* in the clockwise direction with respect to pACYC184.

Media. For both shake flask and fermentation studies, IPTG was added at a final concentration of 1 mM as a gratuitous inducer of the *lac*-based expression vectors that were used in this study. Ampicillin was added at a final concentration of 100 μg/ml as selective pressure for plasmids with ColE1 replicons and chloramphenicol was added at a final concentration of 20 μg/ml as selective pressure for plasmids with P15A replicons.

For shake flask studies, LB Miller broth was used. Samples of 2 mL volume were inoculated from a plate and were grown for approximately 12 hours in LB Miller broth containing ampicillin and chloramphenicol. These samples were diluted 1:200 into 50 ml of fresh LB Miller broth containing ampicillin and chloramphenicol, and allowed to grow until an OD_{550} of 0.5 was reached. Cultures were then diluted to an OD_{550} of 0.1 using 100 mL of fresh LB Miller broth containing ampicillin and chloramphenicol. IPTG was added and samples were processed at regular intervals and assayed for either β-galactosidase or catechol 2,3-dioxygenase activities.

For fermentation studies, the media contained: 30 g/L glucose, 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 5 g/L Na_2HPO_4, 1.5 g/L KH_2PO_4, 1.0 g/L NH_4Cl, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 20 mg/L $CaCl_2 \cdot 2H_2O$, 1.0 mg/L biotin. Samples of 10 mL volume were inoculated from a plate and were grown for approximately 10 hours in the fermentation media containing ampicillin and chloroamphenicol. The 10 mL volume was used to inoculate a 100 mL shake flask containing the identical fresh media. Each shake flask was permitted to grow for 5-6 hours before using to inoculate the fermenter. IPTG was added after inoculation of the fermenter. Batch fermentations were conducted in 2L fermenters (New Brunswick

Scientific Co, New Brunswick, NJ) using 1.6 L of media at a controlled temperature of 37°C, pH of 7.0 and agitation of 800 rpm. Sterile, filtered air was sparged continuously at a rate of 3 L/min. Either 10% NaOH or 2M Na_2CO_3 as a base and 10% H_2SO_4 as an acid were used to control the pH.

Enzyme assays. β-galactosidase assays were performed as described by A. Pardee et al., J. Mol. Biol. 1:165-178 (1959) and J. Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y (1972). One enzyme unit (EU) of β-galactosidase is defined as the quantity of enzyme which produces one μmole of ortho-nitrophenol per minute at 30°C and pH 7. Ortho-Nitrophenol has an absorptivity of 7.5 absorbance units/mM for a 1.0 cm light path. Catechol 2,3-dioxygenase assays were performed as described by Sala-Trepat and Evans (European J. Biochem. 68:69 (1990)) and M. Zukowski et al. (Proc. Natl. Acad. Sci. U.S.A 80, 1101-1105 (1983)). One enzyme unit (EU) of catechol 2,3-dioxygenase is defined as the quantity of enzyme which converts one μmole of catechol to 2-hydroxymuconic semialdehayde per minute at 30°C and pH 8. Catechol has an absorptivity of 36 absorbance units/mM for a 1.0 cm light path. Pyruvate carboxylase activity was measured by the coupled method of J. Payne et al. (J. Gen. Microbiol. 59:97-101 (1969)). One enzyme unit (EU) of pyruvate carboxylase is defined as the quantity of enzyme which converts one μmole of pyruvate to oxaloacetate per minute at 30°C and pH 8.

Analytical methods. The cell growth was monitored by measuring the optical density (OD) (DU-650 spectrophotometer, Beckman Instruments, San Jose, CA) at 550 nm, and this measurement was used to correlate to the dry cell concentration. Glucose and low molecular weight fermentation products were analyzed by high-pressure liquid chromatography using a Coregel 64-H ion-exclusion column as described by Eiteman et al. (Anal. Chim. Acta. 338:69-75 (1997)).

RESULTS AND DISCUSSION

In the first experiment, the plasmid pTrc99A-*pyc*, which overproduces pyruvate carboxylase, and the control plasmid pTrc99A were transformed into the *E. coli* strain MG1655 pACYC-*lacZ* which overproduces β-galactosidase. The resulting strains, MG1655 pTrc99A pACYC-*lacZ* and MG1655 pTrc99A-*pyc* pACYC-*lacZ*, were simultaneously grown in rich media and samples were removed for analysis of β-galactosidase activity. The results are shown in Fig. 12. For both strains, as cell growth occurred and higher optical densities were recorded, increasing β-galactosidase activity was observed. At the point at which the optical density reached about 1.70 absorbance units, the activity of β-galactosidase reached 4.65 EU/mL in the shake flask containing the pTrc99A-*pyc* plasmid, whereas the activity had reached only 2.88 EU/mL in the shake flask containing the pTrc99A plasmid. In this case, the presence of the *pyc* gene encoding for pyruvate carboxylase increased the synthesis of active β-galactosidase by 61% on the basis of normalized cell density.

In the second study, the plasmid pTrc99A-*pyc* which overproduces pyruvate carboxylase and the control plasmid pTrc99A were transformed into the *E. coli* strain MG1655 pACYC-*xylE* which overproduces catechol 2,3-dioxygenase. The resulting strains, MG1655 pTrc99A pACYC-*xylE* and MG1655 pTrc99A-*pyc* pACYC-*xylE*, were simultaneously grown in rich media and samples were removed for catechol 2,3-dioxygenase assays. The results are shown in Fig. 13. As cell growth occurred and higher optical densities were recorded, increasing catechol 2,3-dioxygenase activity was observed. At the point at which the optical density reached about 1.70 absorbance units, the activity of catechol 2,3-dioxygenase reached 3.28 EU/mL in the shake flask containing the pTrc99A-*pyc* plasmid, whereas the activity reached only 2.40 EU/mL in the shake flask containing the pTrc99A plasmid. In this case, the presence of the *pyc* gene encoding for pyruvate carboxylase increased the synthesis of active catechol 2,3-dioxygenase by 37% on the basis of normalized cell density.

Because the shake flask experiments were so successful, we next grew the

β-galactosidase-producing strains in a 2 L fermenter, where the temperature, pH and agitation control would enable higher cell concentrations, and thus yield greater protein production. The first pair of fermentations (i.e., one with the *pyc* gene, one without) was conducted using 10% NaOH as the base, and Fig. 14 shows the results. Both the pTrc99A and the pTrc99A-*pyc* strains grew at a similar rate, achieving a cell concentration of about 4.5 g/L after six hours of fermentation. (This cell concentration corresponds to an optical density of 15.0). Both the pTrc99A and the pTrc99A-*pyc* strains yielded about 10-15 EU/mL of β-galactosidase after 4 hours of fermentation. However, beyond this time the strain containing the *pyc* gene continued to generate β-galactosidase, while the production of this enzyme by the strain that did not contain the *pyc* gene almost halted. At 12 hours, the activity of β-galactosidase generated by the pTrc99A-*pyc* strain was 30.6 EU/mL, while the activity generated by the strain without the *pyc* gene was only 12.9 EU/mL. The activity of β-galactosidase was therefore 137% greater in the fermentation using the strain with the *pyc* gene than in the fermentation using the strain without the *pyc* gene. The presence of pyruvate carboxylase was confirmed by an enzyme assay.

We were interested in learning whether the glucose was consumed at similar rates during these two fermentations, and whether additional low molecular weight chemicals were synthesized by the cells, particularly acetate. Therefore, glucose and acetate concentrations were measured during these 2 L fermentations, and Fig. 15 shows these results. The strain without *pyc* consumed most of the glucose after 6 hours of fermentation. However, the strain with *pyc* consumed glucose more slowly, with about 7 g/L glucose remaining after 6 hours and about 3 g/L still remaining after 12 hours. As shown in Fig. 15, the slower glucose consumption rate in the pTrc99A-*pyc* strain did not result in lower cell growth rate.

Since pyruvate carboxylase uses carbon dioxide as a co-substrate in the conversion of pyruvate to oxaloacetate, we investigated whether using sodium carbonate instead of sodium hydroxide as the base added to control the pH would affect our results. The same fermentations which had been accomplished with sodium hydroxide were completed with this new base, and Figs. 16 and 17 shows

the results. The results indicate that the pTrc99A strain grew slightly more quickly than the pTrc99A-*pyc* strain. At 4 hours of fermentation, both strains produced β-galactosidase activity of about 20 EU/mL. However, after 4 hours, the pTrc99A-*pyc* strain produced β-galactosidase activity more quickly. At 12 hours, glucose was essentially exhausted for the pTrc99A strain which had produced only 82.0 EU/mL of β-galactosidase activity. At 15 hours, on the other hand, the pTrc99A-*pyc* strain still had 2 g/L glucose, and had produced 131.8 EU/mL of β-galactosidase activity. Thus, at the time glucose was exhausted, the activity of β-galactosidase was 61% greater in the fermentation using the strain with the *pyc* gene than in the fermentation using the strain without the *pyc* gene.

Regardless whether the cells overexpressed pyruvate carboxylase, the fermentations using carbonate as a base resulted in higher yields than the analogous experiments using hydroxide as a base, presumably because major reactions in the cell require carbon dioxide. And, regardless of what base was used in the fermentations, pyruvate carboxylase activity lengthened the time over which the glucose was consumed, extending the time during which the TCA cycle could function without being limited by the availability of carbon. Glucose was used much more efficiently when the *pyc* gene was present. Therefore, the presence of the *pyc* gene was a very effective means for either fermentation to divert carbon toward oxaloacetate for the enhanced production of protein.

Example IX. Enhanced Yield of a Recombinant Protein in *E. coli* Expressing Pyruvate Carboxylase Using a Defined Minimal Media.

Because minimal defined media is routinely used by companies that manufacture industrial enzymes and proteinaceous drugs in order to minimize process variability and reduce subsequent complications with purifications, we examined the production of the model protein β-galactosidase using an established defined media.

MATERIALS AND METHODS

Bacterial strains and plasmids. The host strain was *E. coli* MG1655. Table 7, Example VIII describes the plasmids which were used to express β-galactosidase and pyruvate carboxylase in this study. Because both the pTrc99A and pACYC184 expression plasmids contain the *lac* promoter, isopropyl-β-thiogalactopyranoside (IPTG) was added for protein induction.

Media and growth conditions All fermentations were conducted in benchtop fermenters (Bioflow III, New Brunswick Scientific, Co., Edison, NJ). Media were modified from that of Horn et al. (Appl. Microbiol. Biotechnol. 46, 524-532 (1996)). Several colonies were used to inoculate 1 mL of Luria-Bertani (LB) broth, incubated at 250 rpm and 37°C for approximately 6 hours before transferring the contents to 100 mL of media C1. Media C1 in 500 mL shake-flasks was used to grow precultures for the fermenters, and contained (per L): $Na_2HPO_4 \cdot 2H_2O$, 6.42g; KH_2PO_4, 3.00g; NH_4Cl, 1.00g; NaCl, 0.50g; citric acid, 2.0g; $Fe_2(SO_4)_3$, 50mg; H_3BO_3, 3.0mg; $MnCl_2 \cdot 4H_2O$, 15mg; disodium EDTA$\cdot 2H_2O$, 9.6mg; $CuCl_2 \cdot 2H_2O$, 1.5mg; $Na_2MoO_4 \cdot 2H_2O$, 2.5mg; $CoCl_2 \cdot 6H_2O$, 2.5mg; $ZnCl_2 \cdot 2H_2O$, 5.0mg; glucose, 20g; $MgSO_4 \cdot 7H_2O$, 0.6g; $CaCl_2 \cdot 2H_2O$, 70mg; ampicillin, 100mg; and chloramphenicol, 20mg. Media components were added in the order listed to prevent precipitation of metals. Precultures were grown at 250 rpm and 37°C to an optical density near 1.5.

The fermentation media C2 contained (per L): KH_2PO_4, 6.00g; $(NH_4)_2HPO_4$, 8.00g; citric acid, 2.1g; $Fe_2(SO_4)_3$, 62.5mg; H_3BO_3, 3.8mg; $MnCl_2 \cdot 4H_2O$, 18.8mg; disodium EDTA$\cdot 2H_2O$, 12mg; $CuCl_2 \cdot 2H_2O$, 1.9mg; $Na_2MoO_4 \cdot 2H_2O$, 3.1mg; $CoCl_2 \cdot 6H_2O$, 3.1mg; $Zn(CH_3COO)_2 \cdot 2H_2O$, 10mg; glucose, 30g; $MgSO_4 \cdot 7H_2O$, 1.5g; $CaCl_2 \cdot 2H_2O$, 70mg; biotin, 1mg; thiamine•HCl, 1mg; ampicillin, 100mg; and chloramphenicol, 20mg. Media components were added in the order listed. Each fermenter was operated at

1000 rpm, 37°C, and with a flowrate of sterile air at 1.2 L/min. 10% NaOH and 10% H_2SO_4 were used to control pH at 6.5-6.7. Cultures were induced with 1.0 mM IPTG when the optical density was approximately 1.5. Samples were stored at –20°C for subsequent analysis.

Analytical methods. During the course of a fermentation, samples were withdrawn for measurement of glucose, biomass, and product concentrations. Cell growth was monitored by measuring optical density at 550 nm (OD_{550}) (DU-650 spectrophotometer, Beckman Instruments, San Jose, CA), and this measurement was correlated with dry cell mass concentration. Glucose and acetate were analyzed by high-pressure liquid chromatography as previously described (M. Eiteman et al., Anal. Chem. Acta, 338, 69-70 (1997)) using a Coregel 64-H ion-exclusion column (Interactive Chromatography, San Jose, CA). CO_2 and O_2 were measured continuously in the fermentation off-gas (Ultramat 23 gas analyzer, Siemens, Munich, Germany).

Enzyme Assays. Aliquots (1.5 mL) of the samples were thawed and centrifuged (6000×g for 20 minutes). The cells were washed and resuspended in 1.0M Tris buffer (pH 8.0), ruptured with a French® Pressure Cell (850 psi) and centrifuged (25,000×g for 20 min at 4°C). The cell-free extract was analyzed for pyruvate carboxylase following the method of Payne and Morris (J. Payne et al., J. Gen. Microbiol., 59, 97 - 101 (1969)). One unit of pyruvate carboxylase activity converts one μmole of pyruvate per minute to oxaloacetate at 30°C and pH 8.

For β-galactosidase activity, aliquots (1.5 mL) were thawed and diluted to an OD_{550} of 0.1 with Luria-Bertani (LB) broth (R. L. Rodriguez et al, Biotechnolog. Bioeng., 57, 71-78 (1983)). Diluted samples were analyzed for β-galactosidase activity following the protocol of A. B. Pardee et al., J. Mol. Biol., 1, 165-178 (1959). One unit of β-galactosidase activity produced one nmole of *o*-nitrophenol per minute at 30 °C and pH 7.

For cellular protein content, samples were thawed and centrifuged (6000×g for 20 minutes at 4°C). The cells from complex media were washed and resuspended in 1.0M Tris buffer (pH 8.0) and then ruptured with a French® Pressure Cell (850 psi) and centrifuged (25,000 × g for 20 minutes at 4°C).

Samples from defined media fermentations were disrupted with Bper II™ Bacterial Protein Extraction Reagent (Pierce). Total cellular protein content was determined for samples taken from defined media fermentations using a BCA™ Protein Assay Kit (Pierce).

RESULTS AND DISCUSSION

Media C2 contained 30 g/L of glucose and 2.18 g/L of ammonium ion (NH_4^+). The relatively high concentration of NH_4^+ was required since there was no other nitrogen source (such as protein hydrolysate) to provide precursors molecules to derive the amino acids. In addition to being a medium which can be used to generate a very high cell density by fed-batch processing, this medium should most clearly demonstrate any difference caused by adding an additional anaplerotic pathway to *E. coli's* central metabolism. Figures 18 and 19 display representative fermentation profiles of *E. coli* using Media C2 for the *pyc*⁻ and *pyc*⁺ strains, respectively. In the strain which did not contain pyruvate carboxylase, β-galactosidase activity leveled off after 10 hours. In contrast, the activity of this model recombinant protein continued to increase after 17 hours in the strain that contained pyruvate carboxylase activity. The presence of pyruvate carboxylase was confirmed by enzyme assay.

The results from multiple fermentations are summarized in Table 8. The strain of *E. coli* having pyruvate carboxylase averaged a 65.5% increase in maximum β-galactosidase activity. Furthermore, the *pyc*-containing strain showed a 24% increase in maximum cell concentration. Unlike the anaerobic fermentations in Example III, the presence of *pyc* resulted in a reduction in specific glucose uptake rate and a slight reduction in specific growth rate. Each of these differences is significant at the α=0.025 level. The pyruvate carboxylase activity in these aerobic fermentations was 0.42 EU/ml as opposed to 0.06 EU/ml in the anaerobic fermentation. The fact that pyruvate carboxylase activity was seven times higher could explain the effect on specific glucose uptake and specific growth rate. Nonetheless, the end result was a significant increase in the maximum activity of model recombinant protein that could be obtained. Although the average maximum acetate concentration in the

fermentations of *E. coli* containing *pyc* was lower than for those fermentations of *E. coli* not containing *pyc*, the difference was not significant. Moreover, since neither strain produced enough acetate to reach the published threshold for acetate inhibition of at least 3.0 g/L (E. Jensen et al., Biotechnol. Bioengin., 36, 1-11 (1990)), one could conclude that acetate did not play a role in inhibiting growth or protein production in this study. Acetate is not significantly correlated to protein production or specific growth rate ($\alpha = 0.10$).

This work has demonstrated that protein production can be increased by adding an additional anaplerotic pathway to aerobically grown cultures of *E. coli*. This study furthermore demonstrates that acetate does not play a direct role in inhibiting recombinant protein production. Since only 1 mg of acetate may provide enough carbon for about 10,000U of β-galactosidase activity, it is possible that a relatively small and unnoticed redirection of carbon from acetate or its precursors to protein contributes to significantly increased activity beyond any inhibitory effect.

Table 8. Comparison of *E. coli* pTrc99A pACYC184-*lacZ* (without *pyc*) and *E. coli* pTrc99A-*pyc* pACYC184-*lacZ* (with *pyc*) for the production of β-galactosidase in defined media. Standard deviations of each measurement appear in parentheses.

Parameter	*E. coli* without *pyc*	*E. coli* with *pyc*
Maximum β-galactosidase activity (U/mL)	63.5 (2.40)	105.1 (3.75)
Specific glucose uptake rate (mmol/L)	5.89 (0.06)	4.06 (0.47)
Specific growth rate (/h)	0.340 (0.024)	0.300 (0.013)
Maximum cell density (g/L)	5.96 (0.60)	7.40 (0.01)
Maximum acetate concentration (g/L)	1.84 (1.58)	1.20 (0.35)

Example X. Using Overexpression Of Pyruvate Carboxylase To Affect Protein Expression In Eukaryotic Cells

This technology can be readily applied in eukaryotic systems using methodologies analogous to those described in Examples VIII and IX. To use this technology in fungal cells such as the yeast *Saccharomyces cerevisiae*, for example, two compatible plasmids are constructed. The first plasmid expresses the pyruvate carboxylase enzyme while the second plasmid expresses the protein whose expression is to be augmented. In *S. cerevisiae* multiple plasmids can coexist in the same cell and each plasmid is selected for using a different positive selection pressure. While antibiotics are usually used to provide selective pressure for plasmids in bacteria, biosynthetic enzymes such as those involved in the synthesis of histidine, leucine, tryptophan, or uracil are usually used to accomplish this in *S. cerevisiae*. Specifically, the *HIS3, LEU2, TRP1,* or *URA3* genes are routinely used.

A number of plasmids are available in yeast that are well suited for the expression of heterologous proteins, such as pG-3 (M. Schena et al., Meth. Enzymol., 194, 389-398 (1991)), pRA-6 (T. Nagashima et al., Biosci. Biotech. Biochem., 58, 1292-1296 (1994)), pTRP11 (K. Kitamoto et al., Agric. Biol. Chem., 54, 2979-2987 (1990)), and pYcDE-1 (G. L. McKnight et al., Proc. Natl. Acad. Sci. USA., 80, 4412-4416 (1983)). The pyruvate carboxylase gene and the gene that encodes the protein to be tested are cloned into two compatible yeast expression vectors which contain different selectable biosynthetic enzymes. The two resulting plasmids are then transformed into a yeast recipient cell that is deficient for both of the biosynthetic enzymes that are being used as selectable markers.

To perform the fermentation tests, a yeast cell that overproduces pyruvate carboxylase and the protein to be tested is compared to a yeast cell that overproduces the protein to be tested but does not also overproduce pyruvate carboxylase. In this case the parental plasmid is substituted for the plasmid that overproduces pyruvate carboxylase. The two yeast cells are grown in a minimal

defined media which allows for selection of the biosynthetic enzymes that are expressed by the two plasmids. Such media typically contains 6.7 g/L of Difco yeast nitrogen base without amino acids and 20 g/L of glucose. Amino acid and base supplements are then added separately. Various salts can be added to facilitate growth to high densities and/or increase the buffering capacity of the medium. Similarly, various feeding strategies can be used to facilitate growth to high cell densities.

It should be noted that the protein or peptide that is overexpressed can be encoded in a chromosomally integrated nucleic acid, although use of a plasmid encoding the polypeptide is preferred.

The complete disclosure of all patents, patent applications, publications, and nucleic acid and protein sequence database records, for example publically accessible information in connection with GenBank deposits, that are cited herein are hereby incorporated by reference. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

WHAT IS CLAIMED IS:

1. A metabolically engineered cell that overexpresses pyruvate carboxylase so as to cause enhanced production of a protein or peptide compared to a comparable cell that does not overexpress pyruvate carboxylase.

2. The metabolically engineered cell of claim 1 selected from the group consisting of a bacterial cell, a plant cell, a protist cell, a fungal cell and an animal cell.

3. The metabolically engineered cell of claim 2 wherein the cell is a bacterial cell.

4. The metabolically engineered cell of claim 3 wherein the bacterial cell is selected from the group consisting of an *E. coli* cell and a *B. subtilis* cell.

5. The metabolically engineered cell of claim 2 wherein the cell is a plant cell.

6. The metabolically engineered cell of claim 2 wherein the cell is a protist cell.

7. The metabolically engineered cell of claim 2 wherein the cell is a fungal cell.

8. The metabolically engineered cell of claim 7 wherein the fungal cell is a yeast cell.

9. The metabolically engineered cell of claim 2 wherein the cell is an animal cell.

10. The metabolically engineered cell of claim 9 wherein the animal cell is selected from the group consisting of a mammalian cell and an insect cell.

11. The metabolically engineered cell of claim 10 wherein the mammalian cell is a selected from the group consisting of a mouse cell and a human cell.

12. The metabolically engineered cell of claim 1 into which a nucleic acid sequence functionally encoding the pyruvate carboxylase has been introduced.

13. The metabolically engineered cell of claim 12 which expresses a heterologous pyruvate carboxylase.

14. The metabolically engineered cell of claim 13 wherein the nucleic acid sequence functionally encoding the heterologous pyruvate carboxylase is derived from *R. etli* or *P. fluorescens*.

15. The metabolically engineered cell of claim 1 wherein expression of the pyruvate carboxylase is inducible.

16. The metabolically engineered cell of claim 1 which comprises an endogenous pyruvate carboxylase gene, and which overexpresses an endogenous pyruvate carboxylase.

17. The metabolically engineered cell of claim 16 wherein the endogenous pyruvate carboxylase gene has been mutated to cause overexpression of the endogenous pyruvate carboxylase.

18. The metabolically engineered cell of claim 1 wherein the cell has been further metabolically engineered to overexpress phosphoenolpyruvate (PEP) carboxylase.

19. The metabolically engineered cell of claim 18 wherein the cell does not utilize PEP to effect transport of glucose into the cell.

20. The metabolically engineered cell of claim 1 in cell culture.

21. The metabolically engineered cell of claim 20 wherein the cell culture is selected from a batch culture, a fed-batch culture, a continuous culture and a perfusion culture.

22. The metabolically engineered cell of claim 3 in a bacterial fermentation.

23. The metabolically engineered cell of claim 7 in a fungal fermentation.

24. A method for enhancing the production of at least one protein or peptide in a host cell comprising:

transforming the host cell with a nucleic acid comprising a nucleotide sequence functionally encoding a pyruvate carboxylase enzyme to yield a metabolically engineered cell capable of enhanced production of the protein or peptide.

25. The method of claim 24 wherein the host cell is selected from the group consisting of a bacterial cell, a plant cell, a protist cell, a fungal cell and an animal cell.

26. The method of claim 24 wherein the nucleotide sequence functionally encodes a heterologous pyruvate carboxylase enzyme.

27. The method of claim 26 wherein the nucleic acid sequence is derived from *R. etli.*

28. The method of claim 26 wherein the nucleic acid sequence is derived from *P. fluorescens.*

29. The method of claim 24 wherein the protein or peptide is a recombinant protein or peptide.

30. The method of claim 24 wherein expression of the pyruvate carboxylase in the host cell is inducible, and wherein the method further includes inducing expression of the pyruvate carboxylase in the metabolically engineered cell.

31. The method of claim 24 wherein the nucleotide sequence functionally encodes an endogenous pyruvate carboxylase enzyme.

32. The method of claim 24 wherein the metabolically engineered cell has been further metabolically engineered to overexpress PEP carboxylase.

33. The method of claim 24 further comprising culturing or fermenting the metabolically engineered cell for a time and under conditions to produce a protein or a peptide.

34. The method of claim 33 wherein the activity or amount, per cell, of the protein or peptide produced in the metabolically engineered cell in the culturing step is increased relative to the activity or amount, per cell, of the protein or peptide produced under similar culture conditions by a culture of comparable cells that do not overexpress pyruvate carboxylase.

35. The method of claim 33 wherein the cell density attained by the metabolically engineered cells in the culturing step is higher than the cell density attained under similar conditions by a culture of comparable cells that do not overexpress pyruvate carboxylase.

36. The method of claim 33 wherein the culturing step is carried out for a time defined by the length of time during which the yield of the protein or peptide continues to increase, and wherein the length of the culturing step is longer than the length of the culturing step, similarly defined, of a culture of comparable cells that do not overexpress pyruvate carboxylase.

37. The method of claim 33 wherein the yield of the protein or peptide achieved for the metabolically engineered cells during the culturing step is higher than the yield of the protein or peptide achieved under similar culture conditions by a culture of comparable cells that do not overexpress pyruvate carboxylase.

38. The method of claim 33 further comprising isolating the protein or peptide.

39. A method for enhancing protein or peptide production in a host cell comprising:

mutating a pyruvate carboxylase gene of a host cell that produces a yield of protein or peptide such that the host cell overexpresses pyruvate carboxylase to yield a metabolically engineered cell capable of enhanced production of the protein or peptide.

40. The method of claim 39 wherein the host cell is selected from the group consisting of a bacterial cell, a plant cell, a protist cell, a fungal cell and an animal cell.

41. A method for making a protein or peptide comprising:

culturing a metabolically engineered cell that overexpresses pyruvate carboxylase for a time and under conditions to produce the protein or peptide.

42. The method of claim 41 wherein the metabolically engineered cell is selected from the group consisting of a bacterial cell, a plant cell, a protist cell, a fungal cell and an animal cell.

43. The method of claim 41 wherein the cell overexpresses an endogenous pyruvate carboxylase.

44. The method of claim 41 wherein the cell overexpresses a heterologous pyruvate carboxylase.

45. The method of claim 44 wherein the pyruvate carboxylase is derived from *R. etli.*

46. The method of claim 44 wherein the pyruvate carboxylase is derived from *P. fluorescens.*

47. The method of claim 41 wherein the protein or peptide is a recombinant protein or peptide.

48. The method of claim 41 wherein expression of the pyruvate carboxylase in the metabolically engineered cell is inducible, and wherein the method further includes inducing expression of the pyruvate carboxylase in the metabolically engineered cell.

49. The method of claim 41 wherein the metabolically engineered cell has been further metabolically engineered to overexpress PEP carboxylase.

50. The method of claim 41 wherein the activity or amount, per cell, of the protein or peptide produced by metabolically engineered cells in the culturing step is increased relative to the activity or amount, per cell, of the protein or peptide produced under similar culture conditions by a culture of comparable cells that do not overexpress pyruvate carboxylase.

51. The method of claim 41 wherein the cell density attained by the metabolically engineered cells in the culturing step is higher than the cell density attained under similar conditions by a culture of comparable cells that do not overexpress pyruvate carboxylase.

52. The method of claim 41 wherein the culturing step is carried out for a time defined by the length of time during which the yield of the protein or peptide

continues to increase, and wherein the length of the culturing step is longer than the length of the culturing step, similarly defined, of a culture of comparable cells that do not overexpress pyruvate carboxylase.

53. The method of claim 41 wherein the yield of the protein or peptide achieved for the metabolically engineered cells during the culturing step is higher than the yield of the protein or peptide achieved under similar culture conditions by a culture of comparable cells that do not overexpress pyruvate carboxylase.

54. The method of claim 41 further comprising isolating the expressed protein or peptide.

55. The method of claim 54 wherein the protein or peptide produced is selected from the group consisting of an industrial enzyme, a research or diagnostic enzyme, and a therapeutic protein or peptide.

56. The method of claim 41 further comprising transforming a host cell with a nucleic acid fragment comprising a nucleotide sequence functionally encoding a pyruvate carboxylase enzyme to yield the metabolically engineered cell that overexpresses pyruvate carboxylase.

57. The method of claim 56 wherein nucleotide sequence functionally encodes a heterologous pyruvate carboxylase enzyme.

58. The method of claim 41 further comprising mutating an endogenous pyruvate carboxylase gene of a host cell to yield the metabolically engineered cell that overexpresses pyruvate carboxylase.

59. A metabolically engineered cell that overexpresses PEP carboxylase so as to cause enhanced production of the protein or peptide compared to a comparable cell that does not overexpress PEP carboxylase.

60. The metabolically engineered cell of claim 59 wherein the cell does not utilize PEP to effect transport of glucose into the cell.

61. A metabolically engineered cell that overexpresses PEP carboxylase, wherein the cell does not utilize PEP to effect transport of glucose into the cell.

62. A method for making a protein or peptide comprising:

culturing a metabolically engineered cell that overexpresses PEP carboxylase for a time and under conditions to produce the protein or peptide.

63. The method of claim 62 wherein the metabolically engineered cell does not utilize PEP to effect transport of glucose into the cell.

64. The method of claim 62 further comprising isolating the expressed protein or peptide.

65. The method of claim 62 further comprising transforming a host cell with a nucleic acid fragment comprising a nucleotide sequence functionally encoding a PEP carboxylase enzyme to yield the metabolically engineered cell that overexpresses PEP carboxylase.

66. The method of claim 62 further comprising mutating an endogenous PEP carboxylase gene of a host cell to yield the metabolically engineered cell that overexpresses PEP carboxylase.

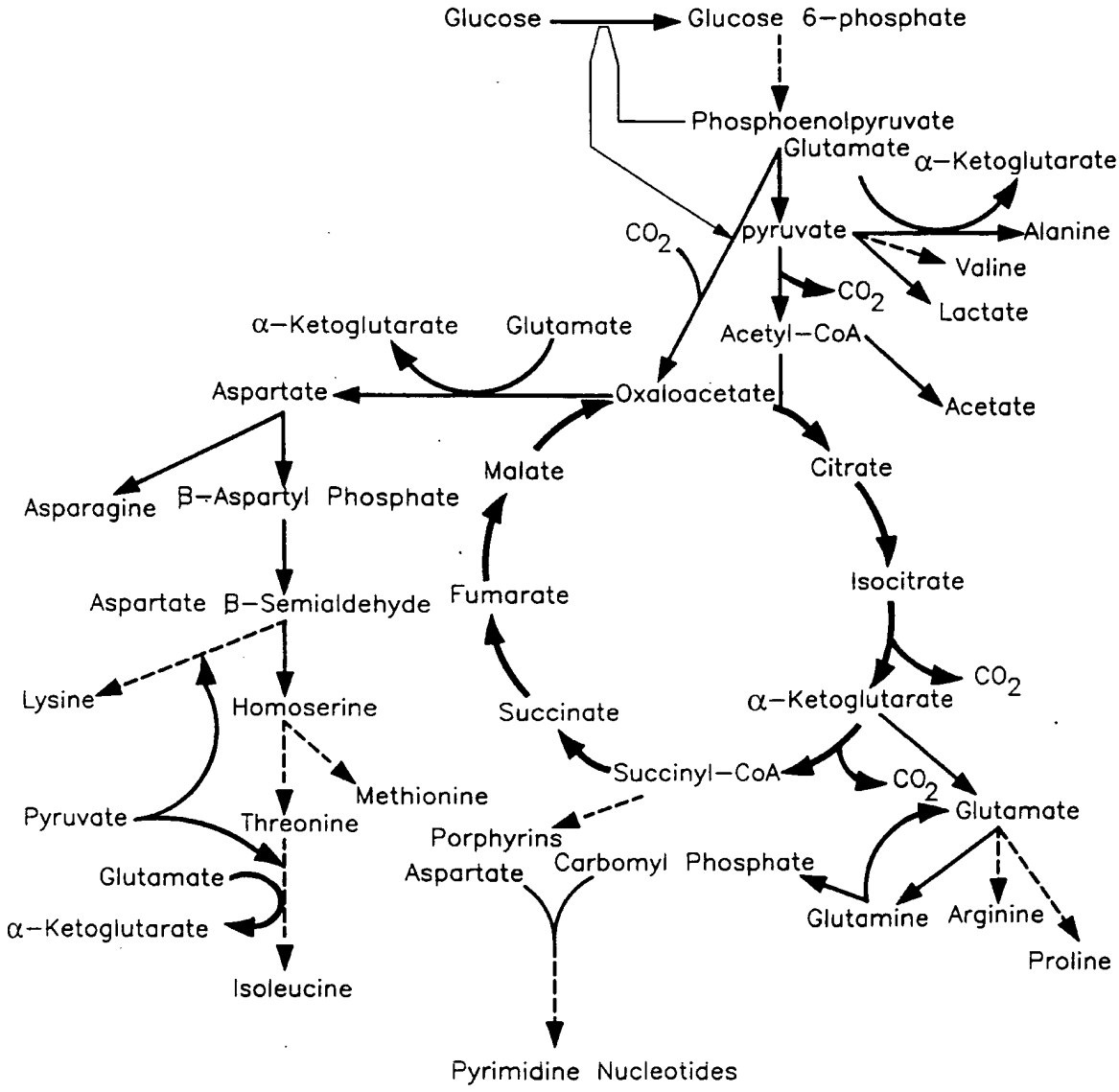
Glucose
Glucose 6-phosphate
Phosphoenolpyruvate
Glutamate
α-Ketoglutarate
pyruvate
Alanine
Valine
Lactate
CO_2
Acetyl-CoA
Acetate
α-Ketoglutarate
Glutamate
Aspartate
Oxaloacetate
Malate
Citrate
Asparagine
B-Aspartyl Phosphate
Aspartate B-Semialdehyde
Fumarate
Isocitrate
Lysine
Homoserine
Succinate
α-Ketoglutarate
Methionine
Succinyl-CoA
Pyruvate
Threonine
Porphyrins
Glutamate
Glutamate
Aspartate
Carbomyl Phosphate
Glutamine
Arginine
Proline
α-Ketoglutarate
Isoleucine
Pyrimidine Nucleotides

FIG. 1

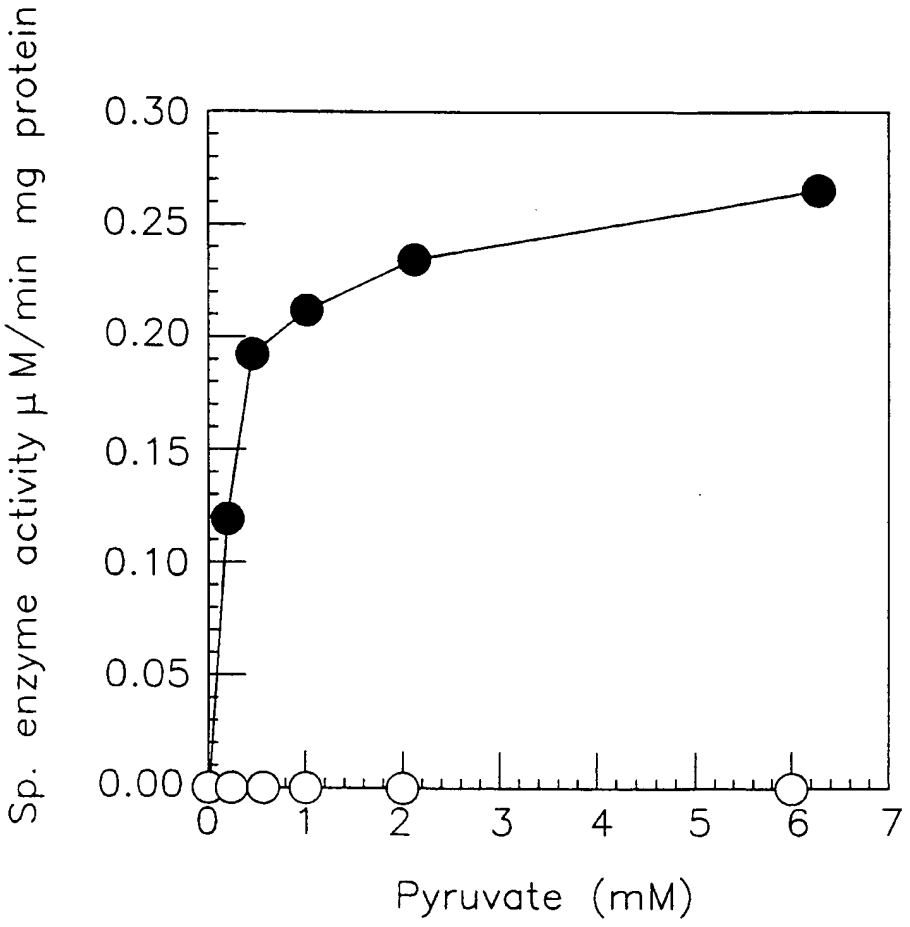
Sp. enzyme activity μ M/min mg protein
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
1
2
3
4
5
6
7
Pyruvate (mM)

FIG. 2

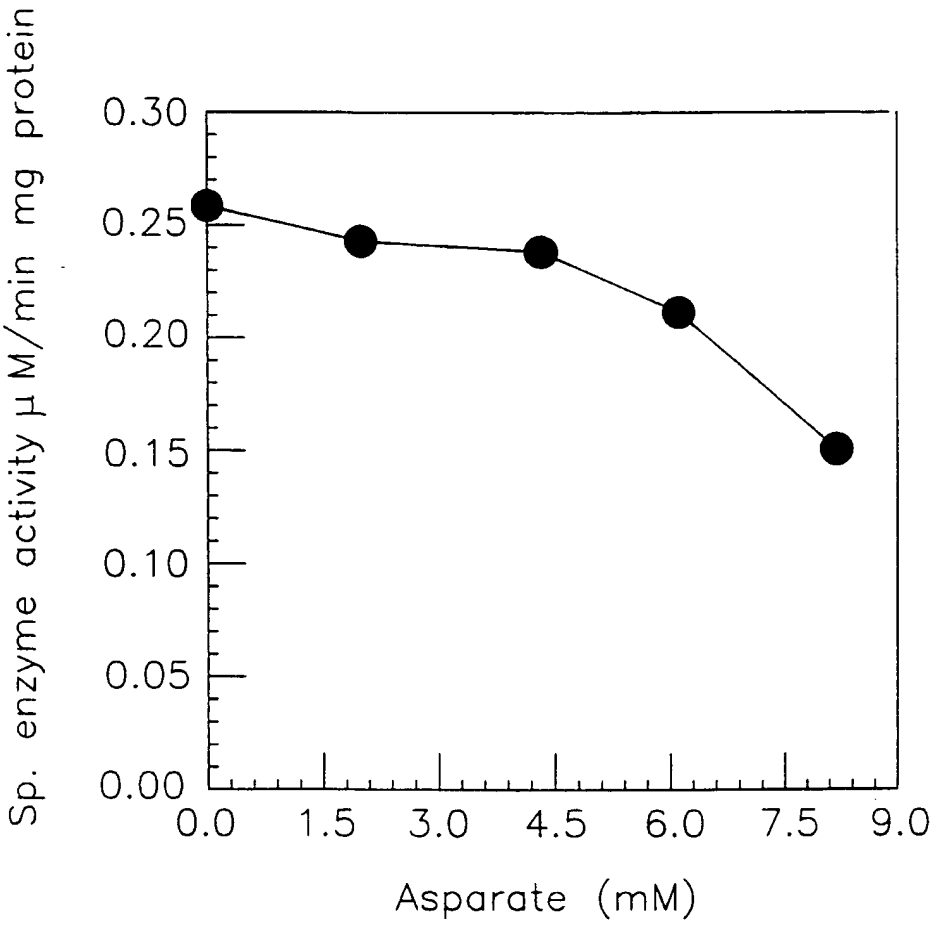
Sp. enzyme activity μ M/min mg protein
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.0
1.5
3.0
4.5
6.0
7.5
9.0
Asparate (mM)

FIG. 3

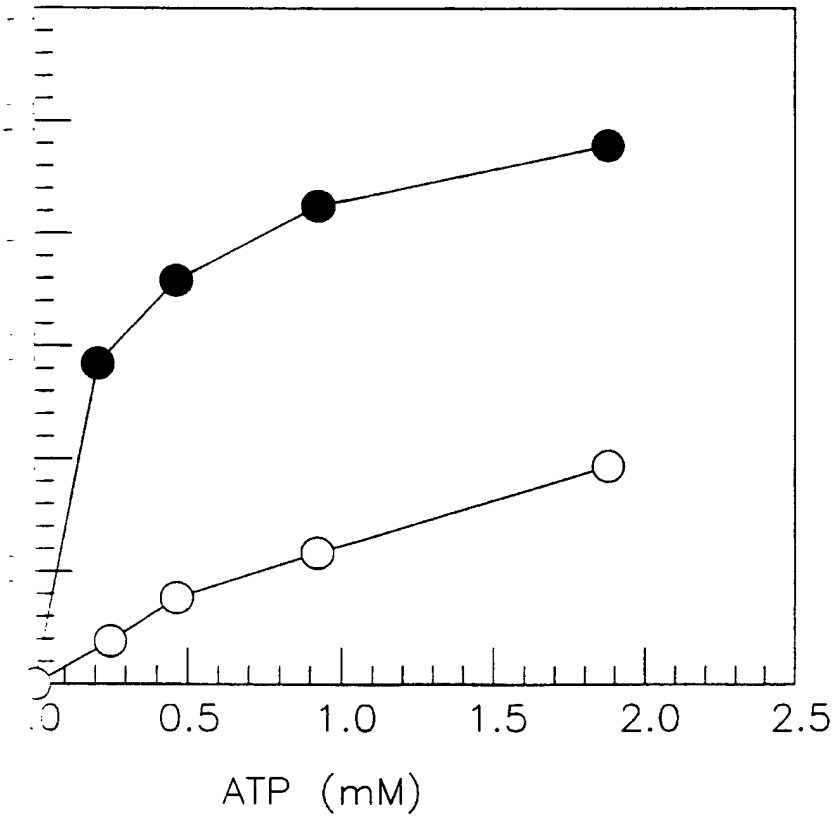

FIG. 4

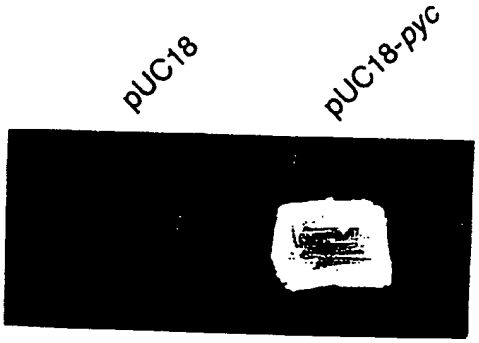

FIG. 5

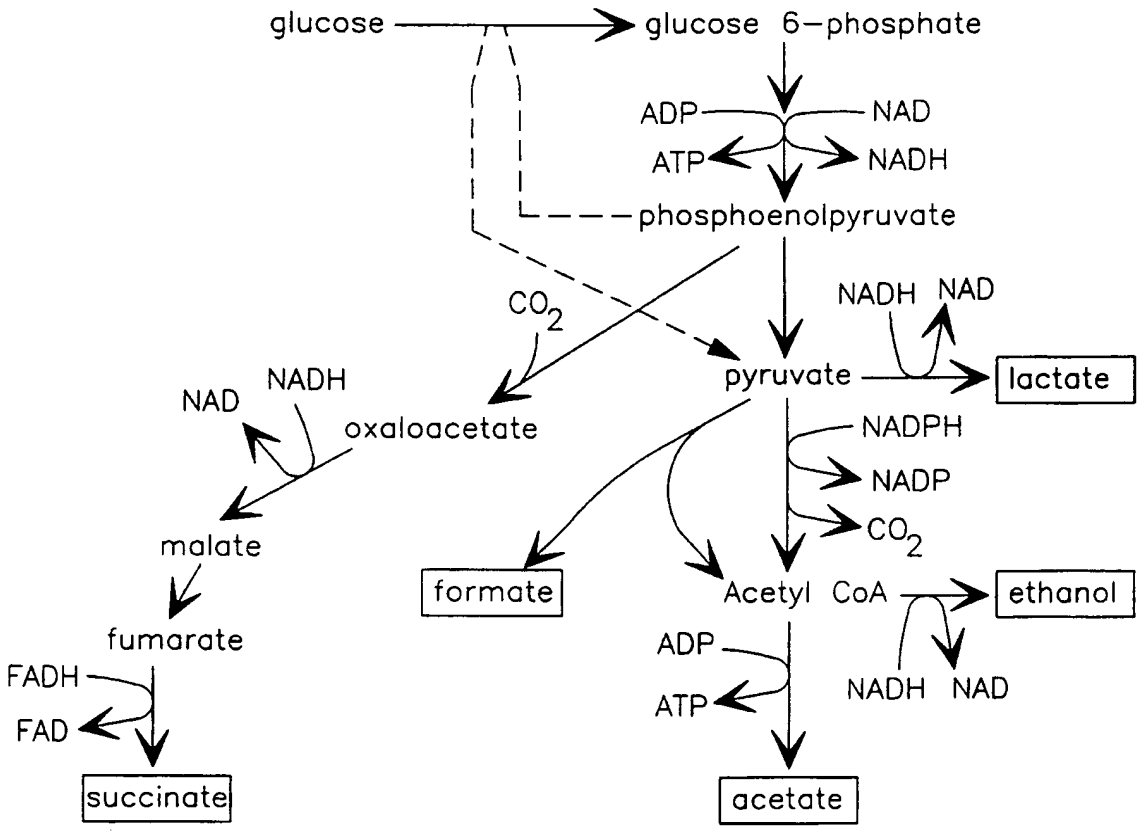
glucose
glucose 6-phosphate
ADP
NAD
ATP
NADH
phosphoenolpyruvate
CO_2
NADH
NAD
pyruvate
lactate
NAD
NADH
oxaloacetate
NADPH
NADP
CO_2
malate
formate
Acetyl CoA
ethanol
fumarate
ADP
FADH
ATP
NADH
NAD
FAD
succinate
acetate

FIG. 6

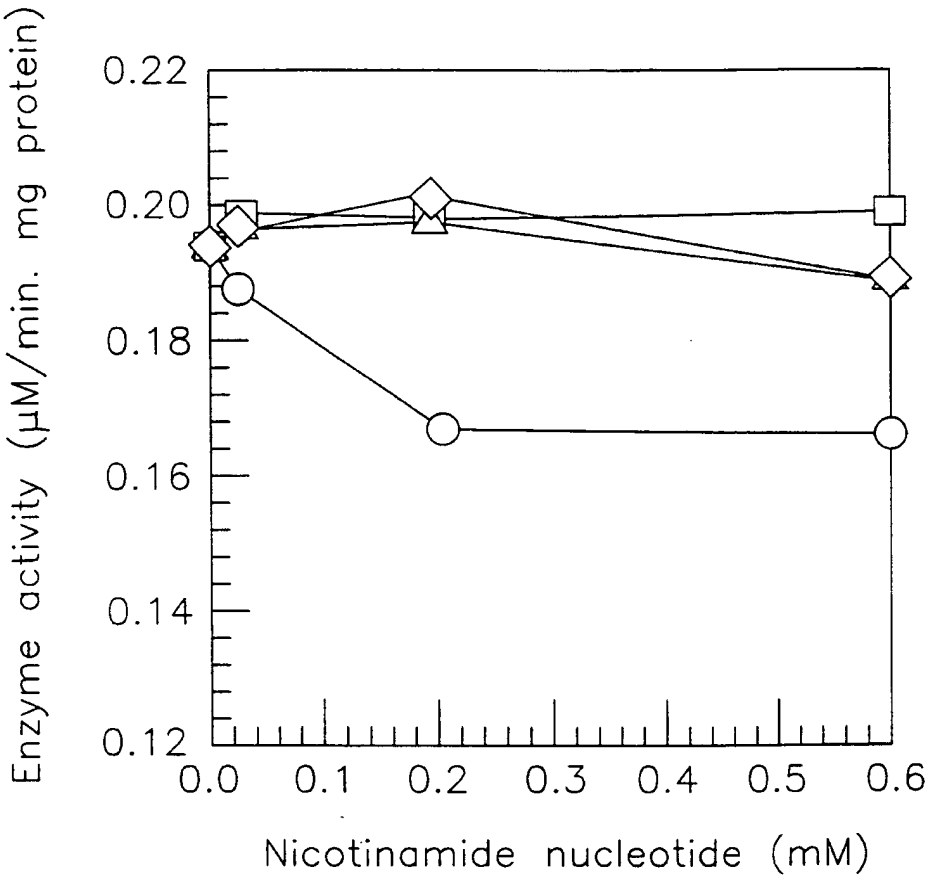
Enzyme activity (μM/min. mg protein)
0.22
0.20
0.18
0.16
0.14
0.12
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Nicotinamide nucleotide (mM)

FIG. 7

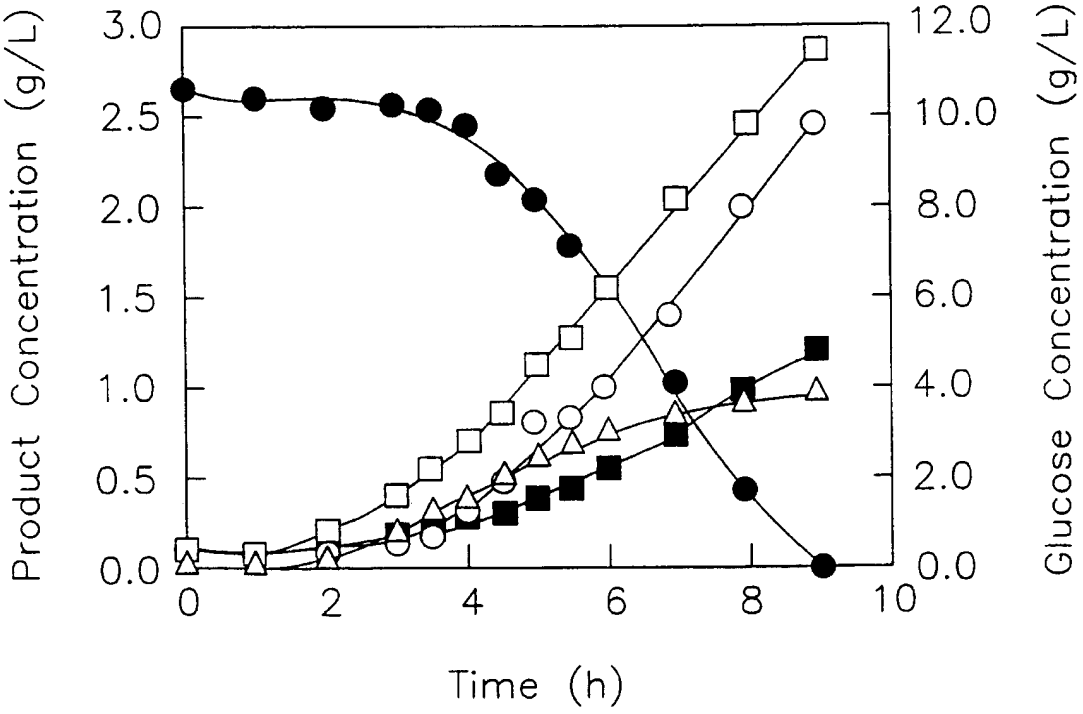
Product Concentration (g/L)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
Time (h)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Glucose Concentration (g/L)

FIG. 8

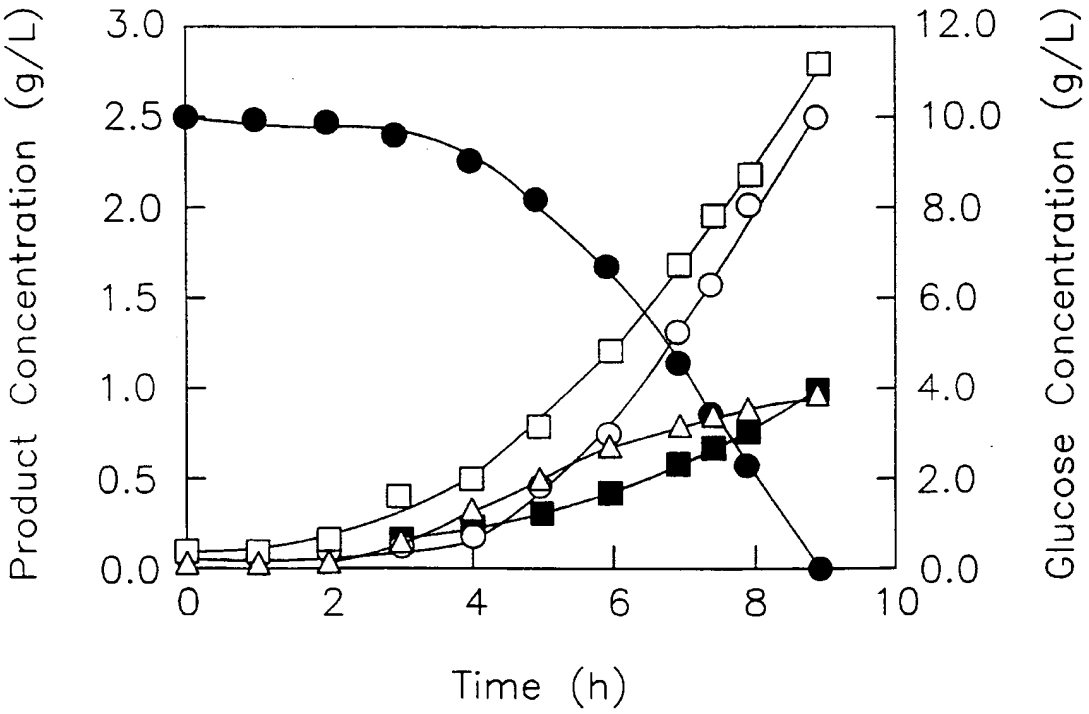
Product Concentration (g/L)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
Time (h)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Glucose Concentration (g/L)

FIG. 9

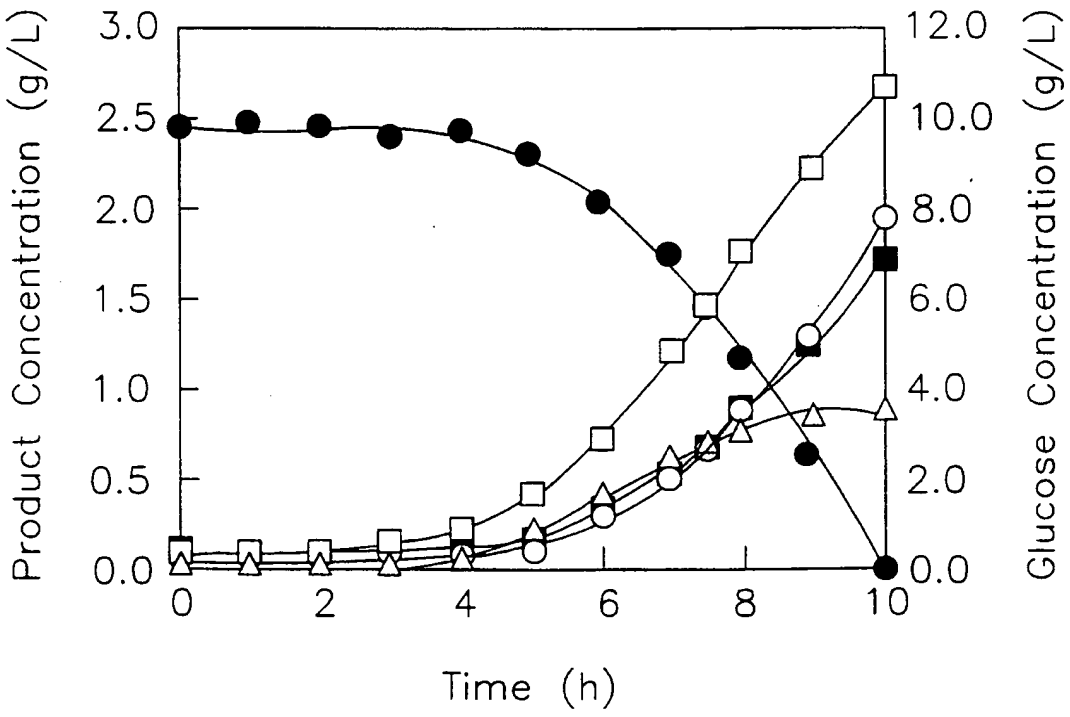
Product Concentration (g/L)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Glucose Concentration (g/L)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
2
4
6
8
10
Time (h)

FIG. 10

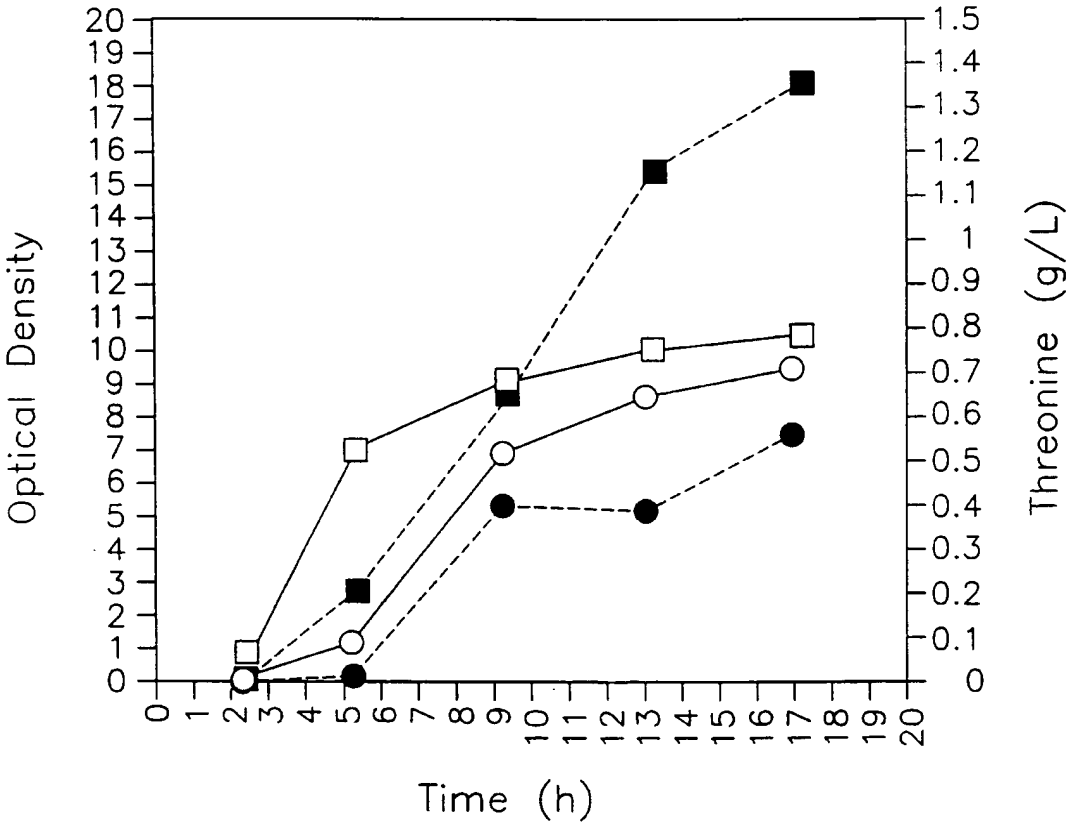
Optical Density
Threonine (g/L)
Time (h)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
1.1
1.2
1.3
1.4
1.5

FIG. 11

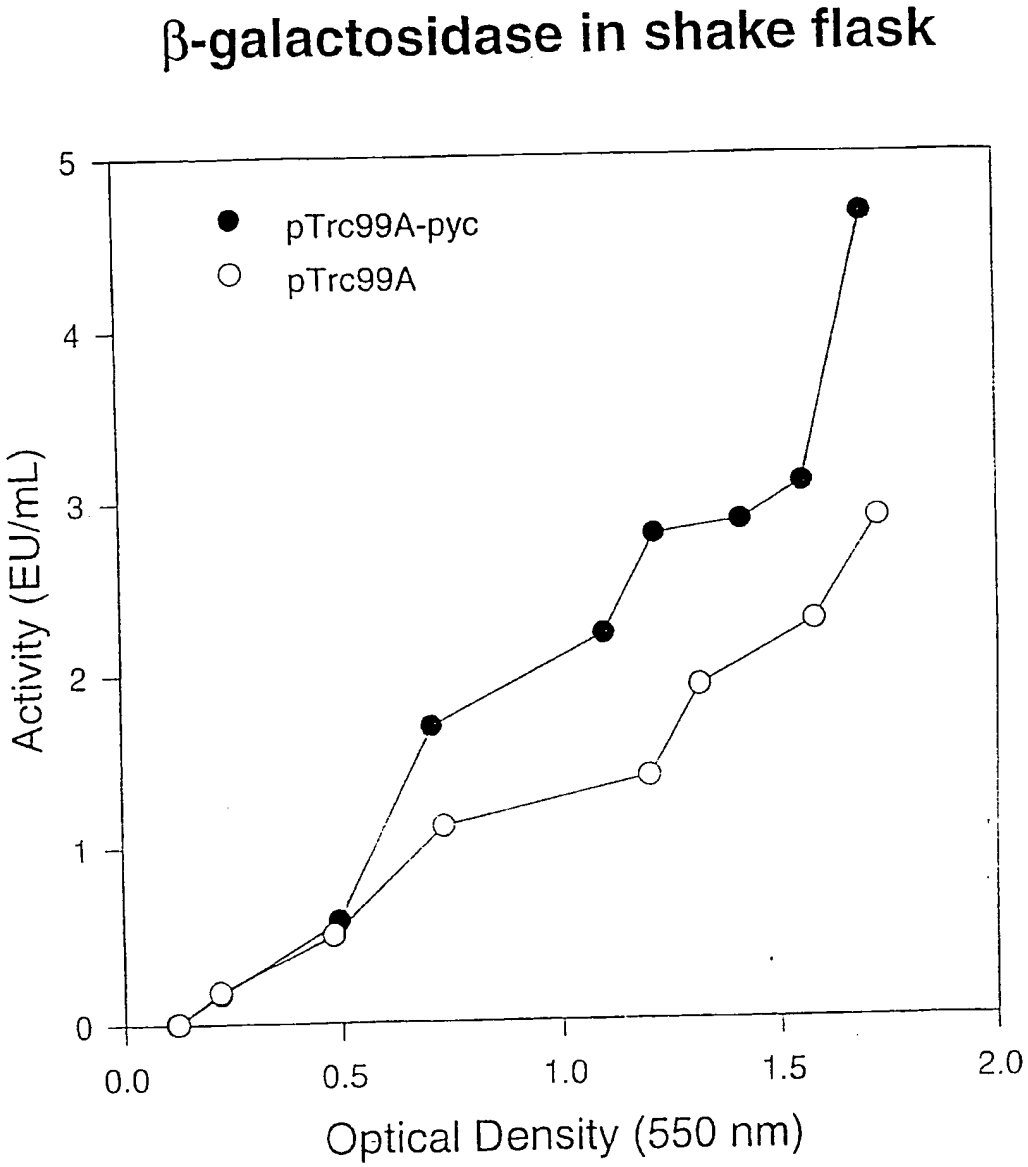
β-galactosidase in shake flask
pTrc99A-pyc
pTrc99A
Activity (EU/mL)
Optical Density (550 nm)
0
1
2
3
4
5
0.0
0.5
1.0
1.5
2.0

FIG. 12

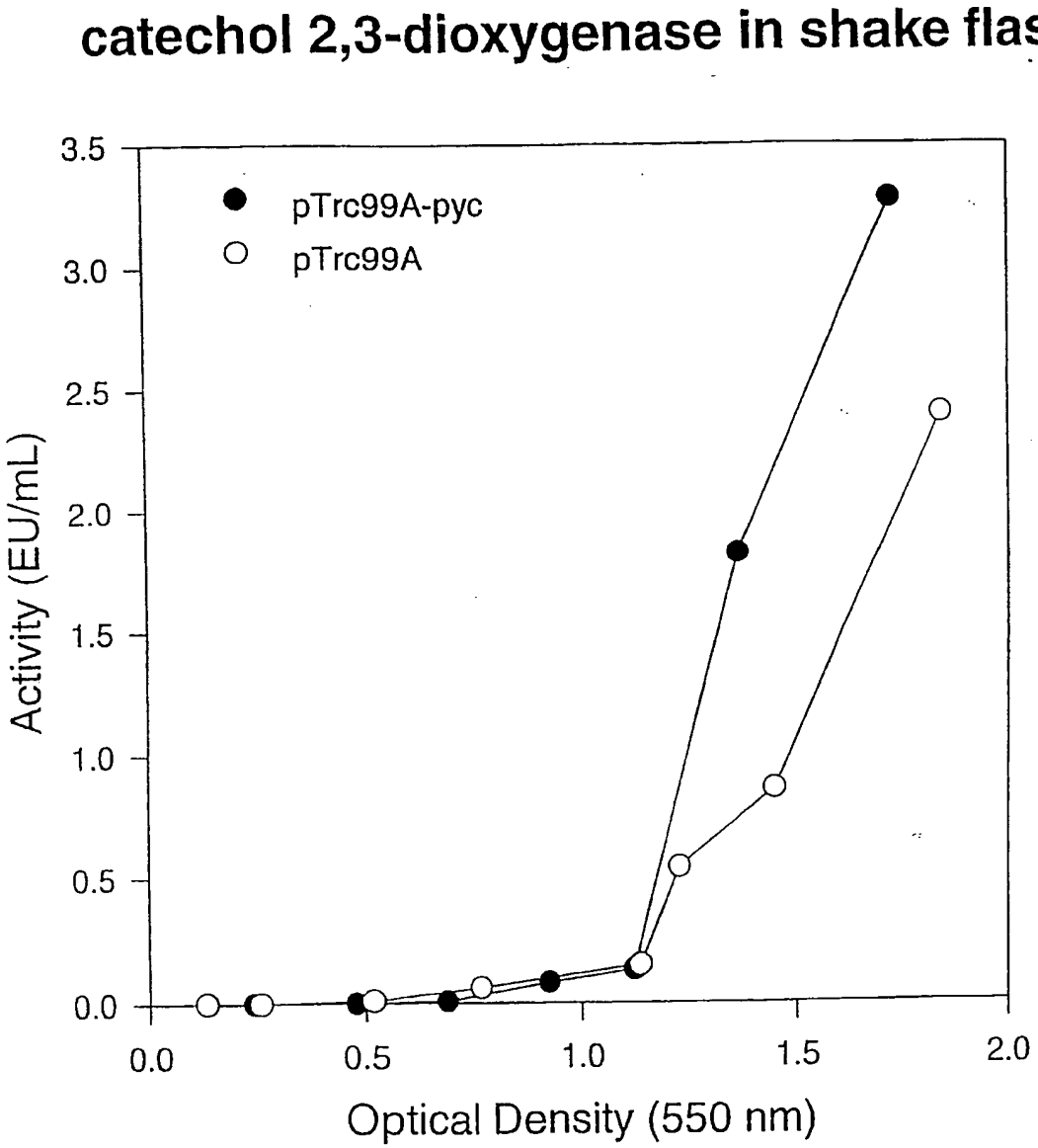
catechol 2,3-dioxygenase in shake flask
pTrc99A-pyc
pTrc99A
Activity (EU/mL)
Optical Density (550 nm)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5

FIG. 13

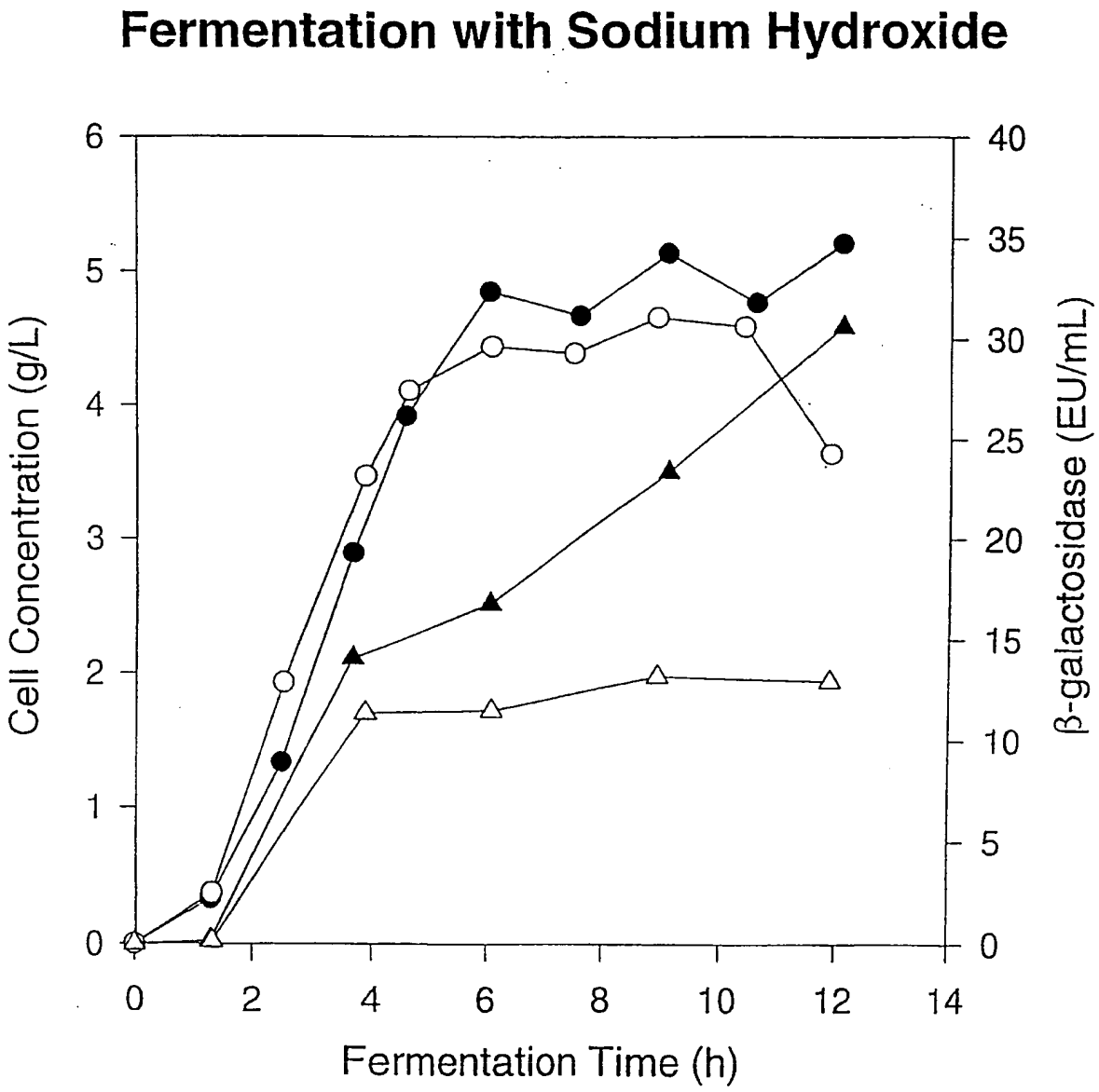
Fermentation with Sodium Hydroxide
Cell Concentration (g/L)
β-galactosidase (EU/mL)
Fermentation Time (h)
0
1
2
3
4
5
6
0
5
10
15
20
25
30
35
40
0
2
4
6
8
10
12
14

FIG. 14

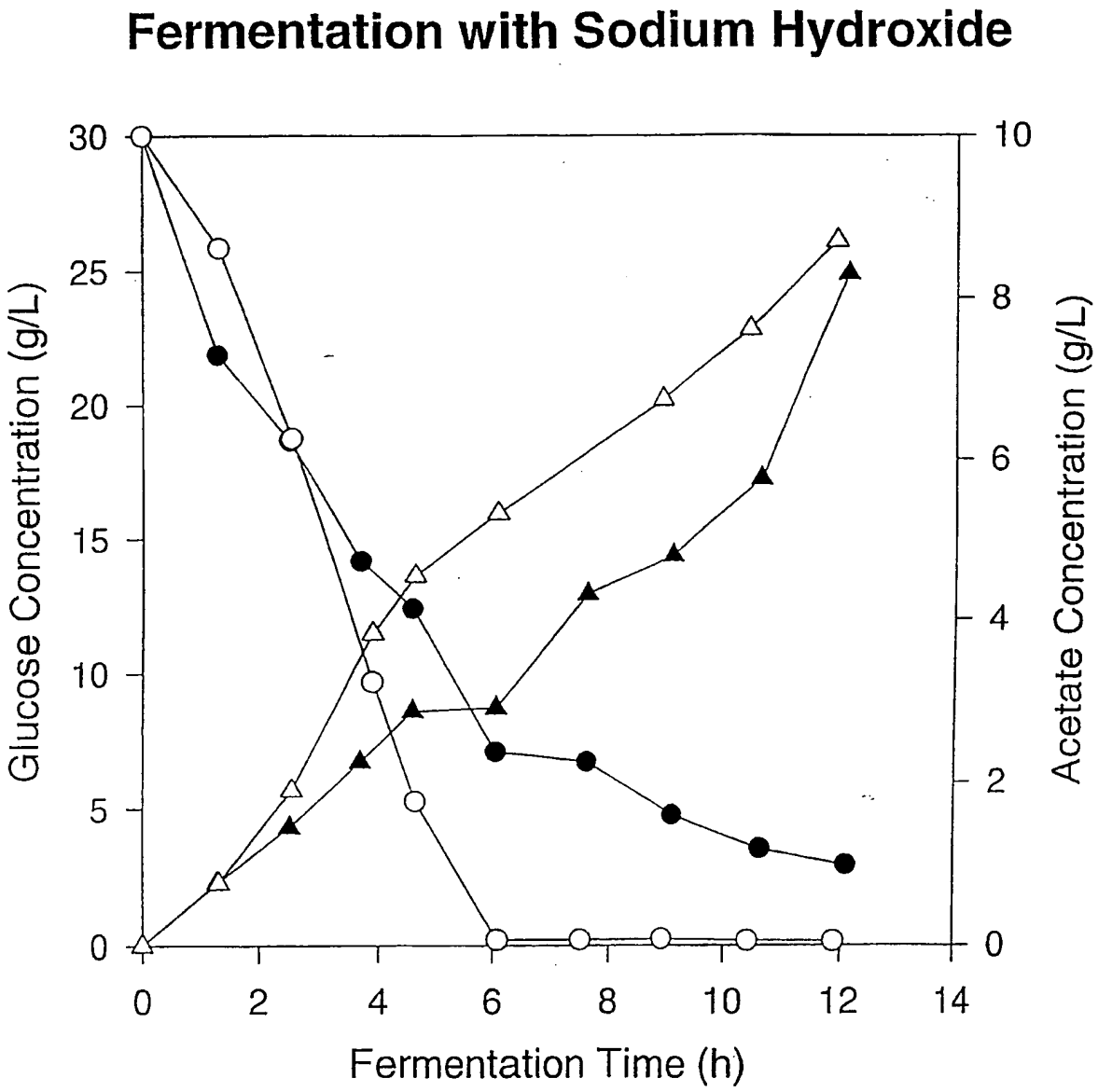
Fermentation with Sodium Hydroxide
Glucose Concentration (g/L)
Acetate Concentration (g/L)
Fermentation Time (h)
0
5
10
15
20
25
30
0
2
4
6
8
10
0
2
4
6
8
10
12
14

FIG. 15

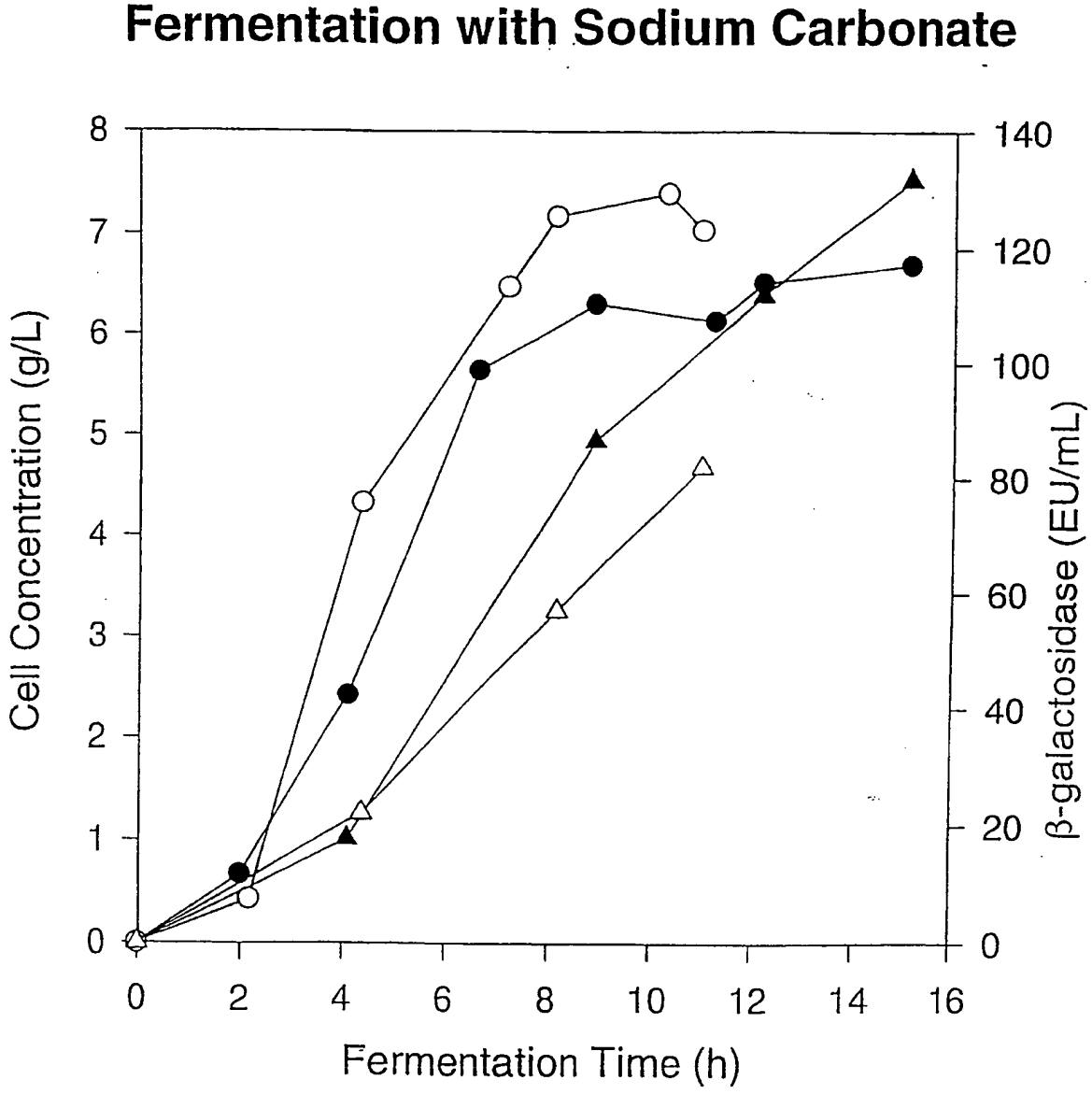
Fermentation with Sodium Carbonate
Cell Concentration (g/L)
β-galactosidase (EU/mL)
Fermentation Time (h)
0
1
2
3
4
5
6
7
8
0
20
40
60
80
100
120
140
0
2
4
6
8
10
12
14
16

FIG. 16

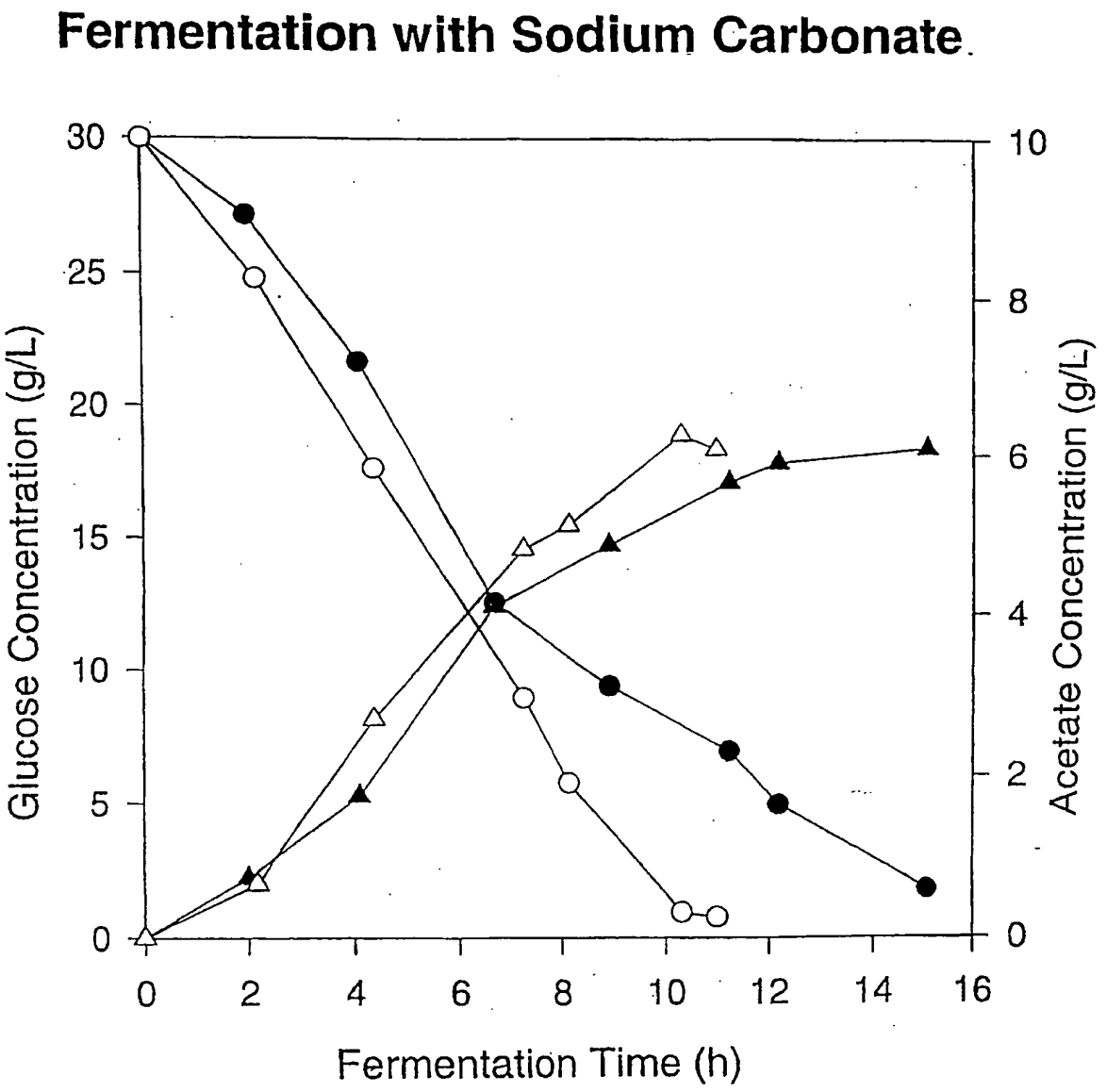
Fermentation with Sodium Carbonate
Glucose Concentration (g/L)
Acetate Concentration (g/L)
Fermentation Time (h)
0
5
10
15
20
25
30
0
2
4
6
8
10
12
14
16
0
2
4
6
8
10

FIG. 17

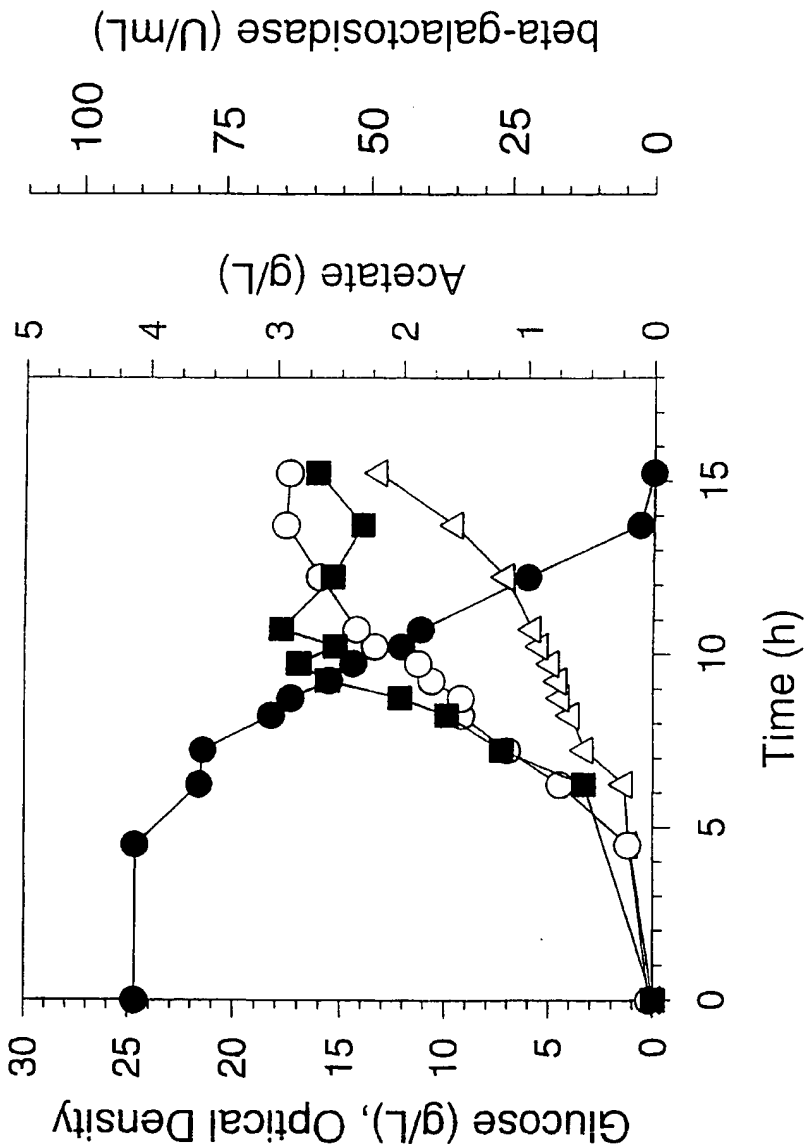
beta-galactosidase (U/mL)
0
25
50
75
100
Acetate (g/L)
0
1
2
3
4
5
Glucose (g/L), Optical Density
0
5
10
15
20
25
30
Time (h)
0
5
10
15

FIG. 18

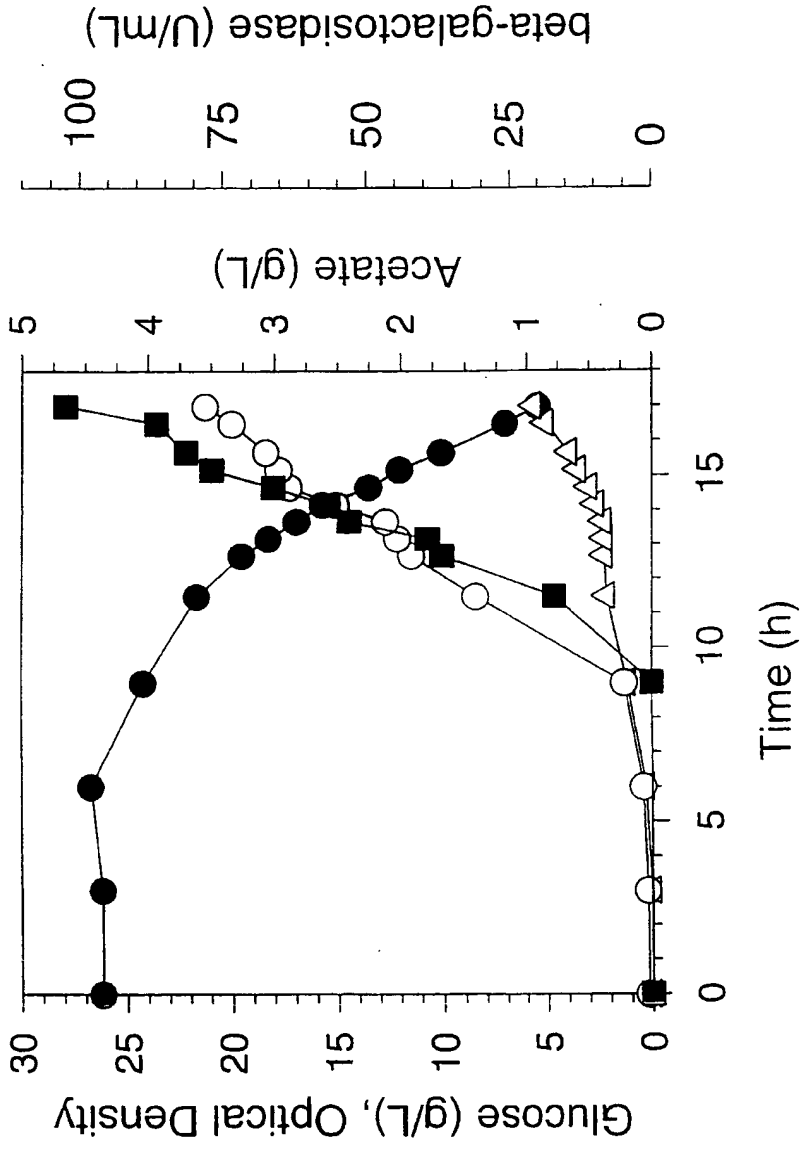
beta-galactosidase (U/mL)
0
25
50
75
100
Acetate (g/L)
0
1
2
3
4
5
Glucose (g/L), Optical Density
0
5
10
15
20
25
30
Time (h)
0
5
10
15

FIG. 19

SEQUENCE LISTING

<110> THE UNIVERSITY OF GEORGIA RESEARCH FOUNDATION, INC., et al.

<120> HIGH YIELD PROTEIN EXPRESSION SYSTEM AND METHODS

<130> 235.00280201

<140> Unassigned
<141> 2000-10-13

<150> 60/159,467
<151> 1999-10-13

<160> 6

<170> PatentIn Ver. 2.1

<210> 1
<211> 49
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: forward primer

<400> 1
tactatggta ccttaggaaa cagctatgcc catatccaag atactcgtt 49

<210> 2
<211> 49
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: reverse primer

<400> 2
attcgtactc aggatctgaa agatctaaca gcctgacttt acacaatcg 49

<210> 3
<211> 47
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 3
tatcatggat ccaggaaaca gctatgacca tgattacgga ttcactg 47

<210> 4
<211> 47

<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 4
tacatactcg agcaggaaag cttggcctgc ccggttatta ttatttt 47

<210> 5
<211> 47
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 5
atcagactgc aggaggtaac agctatgaac aaaggtgtaa tgcgacc 47

<210> 6
<211> 47
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 6
tagcagtggc agctctgaaa gctttgcaca atctctgcaa taagtcg 47